Cell Adhesion Molecules and Cancer Metastasis

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ABSTRACT The adhesive interaction between tumor cells and host cells or the extracellular matrix plays a crucial role in metastasis formation. Therefore, understanding the mechanism controlling metastasis may assist in the development of antimetastatic therapy. We have used synthetic or recombinant polypeptide analogues containing the Arg-Gly-Asp (RGD) sequence found in the functional domains of fibronectin, such as poly(RGD) or CH-271, to regulate the mechanisms involved in cell adhesion during the metastatic process. Poly(RGD) inhibited experimental lung and liver metastasis effectively when coinjected i.v. with various types of tumors. In a model of spontaneous lung metastasis using the B16-BL6 melanoma, repeated administration of this polypeptide before or after surgical excision of the primary tumor resulted in a significant inhibition of tumor metastasis without affecting the growth of the primary tumor and substantially prolonged the survival time of mice. The mechanism responsible for the inhibition of tumor metastasis by the polypeptides is at least partly associated with the ability to interfere with cellular functions such as adhesiveness, motility and invasiveness in the process of metastasis. Combined treatment of the CH-271 fusion polypeptide and anticancer drugs, i.e., anti-adhesion therapy combined with chemotherapy, caused a marked inhibition of lung and liver metastasis of tumors as compared with either treatment alone or with the control. In contrast, the promotion of tumor cell interaction with immune cells via cell adhesion molecules, which differs from the anti-adhesive mechanism, may lead to the induction of anti-tumor immune responses and, consequently, to the inhibition of tumor metastasis. The transfection of the gene of the B7-1 adhesion molecule into tumor cells (B16-BL6 or K1735-M2 melanoma) resulted in the remarkable reduction of lung metastasis caused by the i.v. injection into mice. Immunization of B7-transfected tumor was effective as a tumor vaccine for preventing the metastasis of B7 negative original tumor cells. Thus, the regulation of the adhesive interaction with tumor cells may provide a new and promising approach for the control and prevention of cancer metastasis.

Keywords: Cell adhesion, Metastasis, Invasion, Fibronectin, Arg-Gly-Asp (RGD)

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Abbreviations used are (in alphabetical order): CM-chitin, carboxymethyl chitin; IIICS, type III connecting segment; DOX, doxorubicin; ECM, extracellular matrix; ELAM, endothelial leukocyte adhesion molecule; HSE, hepatic sinusoidal endothelial; ICAM, intercellular adhesion molecule; IL, interleukin; LFA, lymphocyte function-associated antigen; mAb, monoclonal antibody; MHC, major histocompatibility complex; MMP, matrix metalloproteinase; NK, natural killer; SCF-chitin, 6-O-sulfated carboxymethyl chitin; SLeα, sialyl Lewis X; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule.
I. Introduction

Metastasis, one of the major causes of mortality in cancer, is a complex cascade of events involving tumor dissemination from the primary site of growth to distant organs. The pathogenesis of metastases can be subdivided into a variety of sequential steps (Fig. 1): 1) Release from the primary tumor and invasion of the surrounding tissues, 2) entry into vascular or lymphatic circulation, 3) transit in the circulation, 4) arrest in the capillary bed of a distant organ, 5) extravasation from the circulation, and 6) growth at apparently selective sites that are distant from the original tumor site (1–4).

Few cells in a primary tumor can complete all the steps necessary to achieve metastasis. This process is initiated when tumor cells migrate and invade the surrounding tissues. After the cells reach a blood vessel or a lymphatic channel, they penetrate the wall and enter into the circulation (intravasation). Once in the circulation, metastasizing tumor cells encounter various host cells...
(such as platelets, lymphocytes or endothelial cells). This may result in the formation of heterotypic and/or homotypic aggregates, as a result of tumor adhesive interactions between tumor cells and host cells. The formation of tumor-platelet and tumor-tumor aggregates or emboli is an important event in metastasis because there are good correlations between the formation of tumor emboli and the incidence of metastasis (5). Tumor emboli may be arrested nonspecifically into narrow capillaries, and subsequently some of the tumor cells may penetrate the wall (endothelium), degrade the basement membrane and extracellular matrix (ECM), and invade the secondary organ (extravasation). Thus, a large number of adhesive interactions based on cell adhesion molecules or receptors are known to be involved in the metastatic process (Table 1) (2, 4, 6–8). Specific tumor interactions with host cells or components are therefore fundamental events in preferential organ colonization where metastases occur in specific organs and not randomly.

The ability of certain cells within the primary tumor to achieve metastasis is determined by a number of intrinsic properties that include growth rate, adhesiveness, motility, susceptibility to host immune responses, secretion of degradative enzymes, angiogenesis factors and growth factors, antigenicity, and deformability. In addition, some molecules on the surface of metastatic cells serve as receptors or ligands in that they bind complementary molecules on adjacent normal or tumor cells or within the ECM, and they play an important role in tumor invasion and metastasis (Table 1).

These adhesion molecules have been studied intensively in recent years. The primary structures of cell adhesion proteins in the ECM such as fibronectin (9), vitronectin (10) and laminin (11, 12) have been identified by recombinant DNA technology. Common or characteristic core sequences in these molecules have been shown to contribute to adhesion, spreading and migration of cells (13–15). In addition, at least 4 major gene families (Fig. 2), referred to as the immunoglobulin, selectin, cadherin and integrin families, have been identified as adhesion molecules or receptors in cell-cell or cell-ECM interactions (16–20). These molecules have been shown to contribute to the regulatory mechanisms involved in events as diverse as embryogenesis, thrombosis, wound healing and immune processes, as well as metastasis (21). Defining the molecules that mediate the interaction of tumor cells with other cells or with the ECM, and the role of these molecules in metastasis, is of prime importance in achieving control of the cellular functions of metastatic tumor cells. This article focuses on the interaction of metastatic tumor cells with the ECM and describes the effect of peptide analogues derived from fibronectin on tumor invasion and metastasis in vivo and in vitro. It also discusses the place of these analogues in the metastatic cascade in order to gain insight into their regulatory mechanism of action.

### Table 1. Involvement of adhesion molecules at various stages of tumor dissemination

<table>
<thead>
<tr>
<th>Adhesion molecules on tumor cells</th>
<th>Adhesion molecules on other structures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor-tumor</td>
<td></td>
</tr>
<tr>
<td>Cadherin</td>
<td>Cadherin</td>
</tr>
<tr>
<td>N-CAM</td>
<td>N-CAM</td>
</tr>
<tr>
<td>GMP-140</td>
<td>Sugar chain</td>
</tr>
<tr>
<td>CD44</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>LFA-1</td>
<td>ICAM-1</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor-immune cell</td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td>LFA-1</td>
</tr>
<tr>
<td>LFA-3</td>
<td>CD2</td>
</tr>
<tr>
<td>MHC + Antigen</td>
<td>T cell receptor/CD3</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor-platelet (interactions mediated via fibrinogen, fibronectin, vitronectin, von Willebrand factor, etc.)</td>
<td></td>
</tr>
<tr>
<td>β3-Integrin</td>
<td>GPIIb/IIIa</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor-endothelial cell</td>
<td></td>
</tr>
<tr>
<td>VLA-4</td>
<td>CS1, VCAM-1</td>
</tr>
<tr>
<td>LFA-1</td>
<td>ICAM-1</td>
</tr>
<tr>
<td>Mac-1</td>
<td>ICAM-1</td>
</tr>
<tr>
<td>SLε, Leα, Leβ</td>
<td>ELAM-1, GMP-14</td>
</tr>
<tr>
<td>Sulfatide</td>
<td>GMP-140</td>
</tr>
<tr>
<td>CD44</td>
<td>Hyaluronic acid, Chondroitin sulfate proteoglycan</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor-ECM</td>
<td></td>
</tr>
<tr>
<td>VLA-1 (α1β3)</td>
<td>Laminin, Collagen</td>
</tr>
<tr>
<td>VLA-2 (α5β3)</td>
<td>Laminin, Collagen</td>
</tr>
<tr>
<td>VLA-3 (α5β1)</td>
<td>Fibronectin, Laminin, Collagen</td>
</tr>
<tr>
<td>VLA-4 (α4β1)</td>
<td>Fibronectin (CS-1 sequence)</td>
</tr>
<tr>
<td>VLA-5 (α5β3)</td>
<td>Fibronectin (RGD sequence)</td>
</tr>
<tr>
<td>VLA-6 (α5β3)</td>
<td>Laminin</td>
</tr>
<tr>
<td>67 kD receptor</td>
<td>Laminin (YIGSR sequence)</td>
</tr>
<tr>
<td>Glycosaminoglycans</td>
<td>ECM</td>
</tr>
<tr>
<td>(Heparan sulfate, Chondroitin sulfate)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: ECM = extracellular matrix, CS1 = connecting segment 1 of fibronectin, ELAM-1 = endothelial leukocyte adhesion molecule-1, GMP-140 = 140-kD granule membrane protein, ICAM-1 = intercellular adhesion molecule-1, Le = Lewis antigen, LFA = lymphocyte function-associated antigen, MHC = major histocompatibility complex, N-CAM = neural cell adhesion molecule, SLε = sialyl Lewis antigen, VCAM-1 = vascular cell adhesion molecule-1, VLA = very late antigen.
II. Involvement of cell adhesion molecules in the metastatic process

Although a number of epigenetic preconditions leading to the aberrant regulation of cell growth and proliferation must be met, ultimately tumor metastasis may be viewed, in part, as an adhesive disorder. In the multiple steps of tumor metastasis (Fig. 1), many adhesion molecules on tumor cells and host cells or in the components of the ECM may be involved in cell-cell and cell-ECM adhesions (Fig. 2 and Table 1). Some of these interactions may promote tumor growth and organ-specific metastasis. Tumor cells may find appropriate adhesion structures, attach, invade and grow. Because of the sequential steps of the metastatic process, many different adhesion molecules or receptors may be involved directly or indirectly in the organ selectivity of malignant tumor dissemination (Table 1). Therefore, it seems plausible that by interfering with or modulating such adhesive interactions, one might block or suppress blood-borne metastasis.

The initial step of cancer metastasis is the detachment of cells from the primary tumor mass (Step 1 in Fig. 1). In general, when cadherins, Ca\(^{2+}\)-dependent cell-cell-adhesion receptors that bind cells by means of homophilic interactions (17) (Fig. 2), are sufficiently active, cells are not able to disrupt their mutual connections. Therefore, the suppression of cadherin gene expression or the loss of function of expressed cadherin molecules might trigger the release of tumor cells. For example, invasiveness of poorly differentiated epithelial carcinoma that had lost the cell-cell adhesion molecular E-cadherin (22) could be prevented by transfection with E-cadherin cDNA and was again induced by treatment of the transfected cells with anti-E-cadherin monoclonal antibody (mAb) (23, 24). These findings suggest further that E-cadherin acts as an invasion suppressor. Recent studies also demonstrated that the loss of E-cadherin-dependent adhesion of human gastric, prostatic or lung cancer cells was due to the gene mutation of catenin which is associated with the cytoplasmic domain of cadherin and indispensable for the expression of cadherin function (25). On the other hand, the neural cell adhesion molecule (N-CAM), a member of the immunoglobulin superfamily (Fig. 2 and Table 1), plays an important role in the metastatic
process. The disappearance of N-CAM expression in glioma cells has been shown to be accompanied by the development of an increased metastatic capacity (26).

Once in the circulation, metastatic tumor cells encounter various host cells or components (Step 3 in Fig. 1). Metastasizing tumor cells, because of their adhesive properties, interact with host cells such as lymphocytes, natural killer (NK) cells and macrophages that are believed to be particularly important in killing these tumor cells, thus implying that the blood stream provides an incompatible environment for the circulating tumor cells. In contrast, platelets have been reported to enhance the metastatic dissemination of tumor cells at several of the metastatic stages (27). The adhesive interaction between tumor cells and/or platelets may form homotypic or heterotypic cell clumps and aggregations that can subsequently be arrested and extravasated. Such interactions may also lead to an enhancement and stabilization of tumor cell lodgement in capillary vessels by increasing the size of tumor emboli as well as by shielding the tumor cells from the immune response.

There are many reports on the involvement of adhesion molecules in homotypic aggregation of various tumor cells such as galactoside-specific lectin (28–30), a certain carbohydrate (31), lymphocyte function-associated antigen (LFA)-1 (32), CD44 (33), and cadherin. For example, homotypic aggregation of human melanoma cell variants was increased in those cells expressing high levels of CD44, and the aggregation activities were inhibited by the presence of anti-CD44 mAb (33). MAb against endogenous galactose-specific lectin was found to inhibit asialofetuin-induced homotypic aggregation of murine tumor cells and consequently achieve the reduction of tumor lung colonies (29, 30). On the other hand, many animal and human tumor cells can activate and aggregate platelets in vitro (34, 35). Tumor-induced platelet aggregation was inhibited by the Arg-Gly-Asp (RGD)-containing peptides derived from the cell-binding domain of fibronectin, thus indicating that the RGD sequence competitively blocks any interaction between tumor cells, tumor-stimulated platelets (presumably through the 11b/IIla integrin on the surface) and such adhesive proteins like fibrinogen and fibronectin (36–38).

Tumor arrest initiated by tumor-cell-endothelial cell contact followed by extravasation are important steps for the success of hematogenous metastasis (Steps 4 and 5 in Fig. 1). Following arrest, tumor cells must establish stable contacts with the endothelium, induce endothelial cell retraction, migrate, and attach to the subendothelial matrix. Finally, they must proteolytically degrade the subendothelial matrix and extravasate. Thus, tumor cell arrest followed by extravasation into target organs for metastasis formation appears to involve multiple molecular interactions. Recently, several studies have reported that various cytokines such as interleukin (IL)-1 and tumor necrosis factor (TNF) produced by tumor cells activate and induce endothelial cells to express intercellular adhesion molecule (ICAM)-1 (39), endothelial leukocyte adhesion molecule (ELAM)-1 (40) or vascular cell adhesion molecule (VCAM)-1 (41) which normally mediate the adhesion or recruitment of neutrophils and lymphocytes to the stimulated vascular endothelium in the processes of tissue damage or inflammation. This suggests an interesting analogy for tumor metastasis (42). Cytokines may affect the adhesion of malignant tumor cells to the endothelium in a manner similar to that observed in leukocyte-endothelium interaction.

Many epithelial tumor cells have been shown to express sialyl Lewis X (SLex) on the surface, which is well-known as a tumor marker and recognized by selectin families such as ELAM-1 and 140-kD granule membrane protein (GMP-140) (43–45) (Fig. 2 and Table 1). Adhesion of this type of tumor cell to cytokine-treated endothelial cells was inhibited by anti-ELAM-1 mAb (46). Our study has also shown that the treatment with anti mouse ELAM-1 mAb, anti IL-1,3 mAb, or SLex as a ligand for ELAM-1 inhibited the interaction of lymphoma cells with IL-1,3-stimulated endothelial cells (47). It has been also reported that metastatic cell functions such as adhesion and motility were controlled by cell surface glycolipid-glycolipid interactions (48, 49). Production of cytokines including GM-CSF, IL-6, TNF and IL-1 by tumor cells or surrounding tissues may be one of the factors involved in the expression of some cell adhesion molecules, the regulation of tumor growth and organ specific metastasis (50). Thus, adhesive interactions with tumor cells in this process must be controlled precisely and properly by the expression, utilization and combination of different adhesion molecules that can serve as receptors for the circulating/metastasizing tumor cells and promote the subsequent invasive and metastatic spread of tumors.

Nicolson et al. (51) have shown that some lung-metastatic tumor cells adhere preferentially to monolayers of lung-derived endothelial cells, whereas brain-metastatic, liver-metastatic, and ovary-metastatic tumor cells adhere preferentially to endothelial cells isolated from the brain, liver, and ovary, respectively. Similar to the homing of lymphocytes, this process appears to be regulated by organ-specific adhesion molecules expressed on the endothelial cell surface (52, 53). Günther et al. (54) have reported that the splice variant of CD44, a lymphocyte homing receptor detected on lymphocytes, fibroblasts and epithelial cells, is expressed with striking specificity in metastatic pancreatic carcinoma and that overexpression of the molecule in nonmetastatic tumor cells by transfection with the cDNA is sufficient to fully
establish metastatic behavior. Also, the coinjection of
variant-specific mAb with metastatic tumor cells resulted
in the retardation of metastatic spread in vivo (55, 56).

Following the arrest, a tumor cell passes through
several connective tissue barriers that consist of adhesive
molecules such as laminin, fibronectin and other
glycoprotein and proteoglycans (Step 5 in Fig. 1). Tumor
invasion is a complex process involving cell adhesion,
motility (migration), and the secretion of different classes
degradative enzymes. During this process, the inter-
action of tumor cells with ECM components is thought
to be mostly dependent on the presence of integrins, a
superfamily of cell surface αβ heterodimers (19) (Fig. 2).
There are a number of indications that integrins are
altered upon transformation (57–59). Oncogenically trans-
formed cells have been shown to possess the reduced
assembly of fibronectin-rich ECM and the diminished
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N-Methyl-N'-nitro-N-nitrosoguanidine transformation into
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assembly of fibronectin-rich ECM and the diminished
ability to adhere to fibronectin. Human cells subjected to
N-Methyl-N'-nitro-N-nitrosoguanidine transformation into
highly tumorigenic cells showed a significant increase in
the expression of the integrins α6β1, α5β1 and α1β1, which
are receptors for laminin, collagens, and collagen type IV
and laminin, respectively, and the invasive ability through
the basement membranes (59). Transfection of VLA-2
(α2β1) cDNA into rhabdomyosarcoma cells resulted in
marked increase of adhesion to collagen and laminin in
vitro and enhancement of lung metastasis by the i.v.
injection (60). In contrast, the overexpression of α5β1 in-
tegrin in transformed CHO cells, unlike the control CHO
cells, resulted in the loss of tumorigenicity when the
overpressor cells were injected s.c. into nude mice, thus
suggesting that a reduction of this fibronectin receptor is
responsible for the acquisition of anchorage independ-
ence by transformed cells (61).

Preincubation of metastatic murine melanoma cells
with laminin followed by intravenous injections stimu-
lated the formation of pulmonary metastases, whereas a
proteolytic fragment of laminin had the opposite effect
(62). Peptides containing the RGD sequence of the cell-
binding domain in fibronectin, which binds to α5β1 integri,
or the Tyr-Ile-Gly-Ser-Arg (YIGSR) sequence of
laminin, which binds to a high-affinity 67-kD
glycoprotein receptor, showed marked inhibition of lung
metastasis by the coinjection with melanoma cells and the
invasion through reconstituted basement membrane in
vitro (63, 64). Pretreatment ex vivo of tumor cells with a
purified 33-kD heparin-binding fragment of fibronectin,
which promotes tumor cell adhesion by an RGDS-in-
dependent mechanism, effectively inhibited experimental
pulmonary metastases of melanoma or fibrosarcoma (65).
Thus, an adhesive interaction between tumor cells and
ECM components appears to be critical for the formation
of metastasis, and the cell-surface receptor including
integrins may be closely involved in this process (63, 64,
66–68). These findings also suggest that peptides based
on the adhesion molecules that are present in the ECM,
basement membranes or plasma can modulate the
mechanism involved in the metastasising function of
tumor cells.

Coculture of laminin or its fragment which binds to
the cell surface laminin receptor with human melanoma cells
increased the release of type IV collagenase, a Zn2+- de-
pendent matrix metalloproteinase (MMP), but such an
effect by laminin was abolished by mAb against human
laminin receptors (69). These studies suggest that inter-
action of tumor cells with basement membranes including
laminin, which comprises the first step of tumor invasion,
will induce the second step, namely the collagenolytic
dissolution of the basement membrane. Since tissue inhi-
bitor of metalloproteinase (TIMP) is a major negative
regulator of MMP activity and inhibited tumor cell
invasion through the basement membrane (70–72), the
balance between MMP and TIMP may also influence the
invasive potential of tumor cells.

Considering the adhesive interaction of tumor cells
with immune cells such as cytotoxic T cells, macrophages
or NK cells in the metastatic process, the efficient pro-
motion of such interaction may lead to the activation of
effectector cells, induction of anti-tumor immune responses,
and subsequently, to the destruction of target tumor cells.
Cell adhesion molecules involved in the recognition and
destruction of tumor cells by immune cells are mainly in-
cluded in the families of immunoglobulin and integrin
such as major histocompatibility complex (MHC) class I
and II, LFA-1, ICAM-1, B7 and CD28 (Fig. 2 and Table
1). Some cytokines including interferon (IFN)-γ and TNF
are also shown to enhance the expression of ICAM-1 on
tumor cell surface and T cell-mediated destruction of
tumor cells (73). Conversely, in the case of certain lym-
phomas (74), a correlation between the LFA-1 expression
and invasive and metastatic potential has been document-
ed. De novo expression of ICAM-1 during the process of
tumor progression has also been shown to correlate with
increased risk of metastasis in melanoma (75). Therefore,
further study is required to investigate the role of adhe-
sion molecules in the relationship between the ability to
destroy tumor cells and metastatic potentials of tumor
cells in detail.

Based on the two signal theory for T cell activation
(76), the existence/presentation of tumor antigen plus
MHC class I and/or II molecules on antigen-presenting
cells and the costimulatory adhesion molecules on tumor
cells are required for the efficient induction of T cell im-
munity against a tumor. For example, the costimulation
results from an interaction of CD28 or CTLA-4 mol-
ecules on the T cell surface with their ligand, B7, on the
surface of antigen-presenting cells (namely, tumor cells). Several investigators demonstrated that co-expression of B7 molecules with strong viral antigen (E7) on melanoma cells led to the regression of B7 as well as B7+ tumors by a B7-dependent immune response mediated by CD8+ cytotoxic T lymphocytes (77, 78). Thus, immunization of tumor cells transfected with costimulatory adhesion molecules including B7 may be effective for the induction of specific anti-tumor immunity and offer a promising strategy as a tumor vaccine for the treatment of cancer metastasis.

Angiogenesis, the growth of new capillary blood vessels in the host, is a characteristic phenomenon common to most solid malignant tumors. Following establishment of an adequate blood supply, tumor cells will not only grow but also acquire metastatic potential to distant tissues or organs (Steps 1 and 6 in Fig. 1). Tumor angiogenesis is induced by angiogenic factors produced by tumor cells (called tumor angiogenesis factor) which activate and attract endothelial cells (79, 80). This response of microvascular endothelial cells to such soluble factors consists of three major components: increased rate of cell proliferation, a stimulation of endothelial cell migration along a gradient of angiogenic factor and an increased production of proteolytic enzymes (81). Also angiogenesis is not usually active in the normal adult except during wound repair, ovulation and menstruation. Much is known about the molecular and cellular elements of angiogenesis, such as the role of cell adhesion molecules and growth factors in proliferation and migration. During angiogenesis, proliferative endothelial cells become apoptotic in response to antagonists of integrin α5β1, and this leads to the regression of angiogenic blood vessels, thereby blocking the growth of various human tumors (82). Thus the inhibition of angiogenesis (neovascularization) may provide a means to control tumor growth and metastasis (79, 83).

### III. Inhibition of tumor metastasis by fibronectin-derived peptides

Accompanying the rapid progress in analysis of the structures and functions of adhesion molecules and their receptors on the cell surface or within the ECM, several attempts have been made to regulate the mechanism involved in tumor cell interaction with host cells and ECM during the metastatic process. Synthetic peptides derived from ECM components including fibronectin and laminin (63, 64) or mAbs against cell surface integrins, a variant form of CD44, and glycoconjugate Fucα1-2Galβ1-R

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**Fig. 3.** Adhesive interaction of tumor cells with fibronectin and various compounds containing the RGD sequence derived from fibronectin.
Table 2. Effect of polymeric RGD peptide on tumor metastases produced by the i.v. coinjection of various types of tumor cells

<table>
<thead>
<tr>
<th>Tumors</th>
<th>Animals</th>
<th>Peptides</th>
<th>µg/mouse</th>
<th>Tumor metastases</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>B16-BL6 melanoma&lt;sup&gt;a&lt;/sup&gt; (5 x 10&lt;sup&gt;5&lt;/sup&gt;)</td>
<td>C57BL/6</td>
<td>PBS poly(RGD)</td>
<td>500</td>
<td>166 ± 20 (132–286)</td>
<td>27 ± 8 (29–39)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>poly(R, G, D) scrl RGD</td>
<td>500</td>
<td>172 ± 54 (121–245)</td>
<td>171 ± 85 (130–227)</td>
</tr>
<tr>
<td>3LL carcinoma&lt;sup&gt;a&lt;/sup&gt; (3 x 10&lt;sup&gt;5&lt;/sup&gt;)</td>
<td>C57BL/6</td>
<td>PBS poly(RGD)</td>
<td>500</td>
<td>191 ± 54 (117–262)</td>
<td>51 ± 18 (46–112)</td>
</tr>
<tr>
<td>Colon 26 carcinoma&lt;sup&gt;a&lt;/sup&gt; (1 x 10&lt;sup&gt;5&lt;/sup&gt;)</td>
<td>BALB/c</td>
<td>PBS poly(RGD)</td>
<td>100</td>
<td>201 ± 26 (174–232)</td>
<td>81 ± 26 (46–112)</td>
</tr>
<tr>
<td>RAW117-H10 lymphoma&lt;sup&gt;b&lt;/sup&gt; (5 x 10&lt;sup&gt;5&lt;/sup&gt;)</td>
<td>BALB/c</td>
<td>PBS poly(RGD)</td>
<td>1000</td>
<td>2.0 ± 0.05</td>
<td>1.28 ± 0.07</td>
</tr>
<tr>
<td>L5178Y-ML25 lymphoma&lt;sup&gt;c&lt;/sup&gt; (4 x 10&lt;sup&gt;5&lt;/sup&gt;)</td>
<td>CDF1</td>
<td>PBS poly(RGD)</td>
<td>1000</td>
<td>4.02 ± 0.29</td>
<td>2.24 ± 0.46</td>
</tr>
<tr>
<td>A375MM melanoma&lt;sup&gt;a&lt;/sup&gt; (5 x 10&lt;sup&gt;5&lt;/sup&gt;, human)</td>
<td>BALB nude</td>
<td>PBS poly(RGDT)</td>
<td>500</td>
<td>7/7 (100%)</td>
<td>2/6 (33%)</td>
</tr>
</tbody>
</table>

Five animals per group were inoculated i.v. with tumor cells with or without polypeptides indicated above. Mice were killed at the indicated days after tumor inoculation, and the incidence of metastasis was expressed as follows: <sup>a</sup>No. of lung tumor colonies + S.D., <sup>b</sup>Liver weight (g) ± S.D., <sup>c</sup>No. of metastasized mice/No. of tested mice (%).

(49, 84) have been used to inhibit experimental tumor metastasis through interference with tumor-host interactions and metastatizing functions such as attachment and motility (referred to as "anti-adhesion therapy"). Actually, fibronectin-derived peptides such as RGDS (63, 85), CS1 of alternative splicing type III connecting segment (IIICS) (86, 87), and purified 33-kDa trypsin/catheptic heparin-binding fragment (Fig. 3) (65, 88), and laminin-derived peptide Tyr-Ile-Gly-Ser-Arg (YIGSR) have been shown to inhibit lung metastasis when co-injected i.v. with tumor cells (64). Recently, extensive further studies on controlling tumor metastasis have been carried out using synthetic cell-adhesive peptide analogues containing RGDS (Fig. 3) (89) or YIGSR peptides, etc. We have also investigated the effect of some peptide analogues containing the RGD sequence derived from fibronectin on tumor invasion and metastasis.

We first synthesized a unique polymeric peptide, poly(RGD) (Fig. 3), which contains the repetitive structure of RGD for the purpose of augmentation of anti-metastatic effect by RGD-containing oligopeptide (38, 90–92). It is well-known that the introduction of plural peptides (for example, peptide hormones) into carrier protein can augment the activity of the peptide hormone because of the cooperative interaction between the molecules, although at the same time this may also reduce molecular flexibility and mobility and consequently lead to a decrease in the affinity between the peptide and the specific receptors. A markedly high activity of the polymerized functional molecule has been reported as a common phenomenon in the field of polymer catalyst or enzyme-model polymers and has come to be called the "polymer effect". Poly(RGD) inhibited experimental lung or liver metastasis more effectively than the corresponding RGD-containing oligopeptides did when co-injected i.v. into mice with different types of murine or human metastatic tumor cells (Table 2 and Fig. 4) (38, 92). In contrast, a random polypeptide, poly(R,G,D), in which the 3 amino acids Arg, Gly and Asp are randomly arranged, showed no inhibition of lung metastasis of B16-BL6 melanoma cells. Similar results were obtained by using poly(YIGSR),

Fig. 4. Effect of poly(RGD) on lung metastasis of B16-BL6 melanoma cells. C57BL/6 mice were given an i.v. injection of tumor cells (5 x 10<sup>5</sup>) admixed with or without 500 µg of polypeptide. Mice were killed 14 days after the tumor inoculation. Left, control; right, poly(RGD)-treated.
which consists of the repetitive YIGSR sequence derived from the B1 chain of laminin, in place of poly(RGD) (38, 92, 93). These results clearly indicate that polymerization (multivalency) of the RGD sequence is able to augment the inhibition of tumor lung metastasis more effectively than a monovalent unit of RGD peptide and that the effect is RGD-dependent.

We also synthesized polymeric or cyclic Arg-Gly-Asp-Thr (RGDT) peptides with defined sequence repetition, (RGDT)ₙ (ₙ=1 to 11) or cyclo(RGDT)ₙ (ₙ=2 to 4), for examination of their inhibitory effects on tumor metastasis. The use of (RGDT)ₙ and it cyclo(RGDT)ₙ caused significant inhibition of lung metastasis upon coinjection with melanoma cells in proportion to the increase of RGDT sequence repetition, and if did so in a dose-dependent manner (94). (RGDT)ₙ was more effective at inhibiting lung metastasis than cyclo(RGDT)ₙ. The anti-metastatic effect of linear-type (RGDT)ₙ clearly supported the findings using poly(RGD), as described above.

Interestingly, in a therapeutic model of spontaneous lung metastasis using the B16-BL6 melanoma, multiple i.v. administrations of poly(RGD) or its analogue, poly-(RGDT), before or after surgical excision of the primary tumor on day 21, resulted in a significant reduction of lung tumor colonies, but did not affect the growth (size) of the primary tumor (Table 3) (37, 90, 92). Multiple treatment of poly(R,G,D) or RGD tripeptide, however, did not decrease the number of lung tumor colonies. Our model has shown that it takes approximately 7 days for tumor cells to begin metastasising once the animal has been subjected to s.c. inoculation into a hind footpad. The anti-metastatic effect of multiple treatments with poly(RGD), even at an advanced stage of tumor growth (21 days after tumor inoculation), suggests a role for poly(RGD) in suppressing metastasis. This may provide a promising basis for therapy.

Treatment with poly(RGD) substantially prolonged the survival time for mice injected i.v. or s.c. with B16-BL6 cells as compared with treatment with RGD or the random poly(R,G,D) (37, 92). The polypeptide appears to be nonimmunogenic. Since specific antibodies against the polypeptide failed to be induced by immunization in Freund's complete adjuvant containing muramyl dipeptide or multiple injection of the polypeptide, and the polypeptide showed no short term toxicity to the host or in vitro, such characteristics of the polypeptide might be advantageous to the treatment and prevention of cancer metastasis.

VI. Mechanisms for poly(RGD)-mediated inhibition of tumor metastasis

The exact mechanism responsible for the inhibition of tumor metastasis by poly(RGD) or other analogues is still unclear, but may be more complex than a simple interference with the cellular adhesive process of metastasis.

VI-1. Retention of poly(RGD) in the circulation

I.v.-injected, ¹²⁵I-labelled polymeric peptide was biphasically cleared out from the circulation. The clearance rate of the labeled polypeptide during the early phase (within 1 hr) after i.v. injection is almost consistent with that of small GRGDS peptide as reported by Humphries et al. (85), whereas the half-life during the late phase (after 1 hr) is approximately 6 times longer than that of the GRGDS peptide (38). The respective half-life of polypeptide at the early and late phases was approximately 15 min and 6 hr. We also found that

<table>
<thead>
<tr>
<th>Administered i.v. with:</th>
<th>Dose (µg/mouse)</th>
<th>Primary tumor size on day 21 (mm±S.D.)</th>
<th>No. of lung metastases on day 35</th>
<th>Mean±S.D. (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated (PBS)</td>
<td></td>
<td>10±3</td>
<td></td>
<td>75±14 (63–99)</td>
</tr>
<tr>
<td>Poly(RGD) on days 7, 9, 11, 13</td>
<td>100 x 7</td>
<td>10±2</td>
<td>16±10 (3–22)*</td>
<td></td>
</tr>
<tr>
<td>Poly(RGDT) 15, 17, 19</td>
<td>100 x 7</td>
<td>11±2</td>
<td>13±10 (4–28)**</td>
<td></td>
</tr>
<tr>
<td>RGD</td>
<td>100 x 7</td>
<td>11±3</td>
<td>57±18 (44–87)</td>
<td></td>
</tr>
<tr>
<td>RGDT</td>
<td>100 x 7</td>
<td>9±4</td>
<td>53±14 (39–72)</td>
<td></td>
</tr>
<tr>
<td>Untreated (PBS)</td>
<td></td>
<td>106±23</td>
<td></td>
<td>106±23 (73–122)</td>
</tr>
<tr>
<td>Poly(RGD) on days 22, 24, 26, 28,</td>
<td>100 x 7</td>
<td>(10±3)</td>
<td>41±19 (26–73)*</td>
<td></td>
</tr>
<tr>
<td>Poly(RGDT) 30, 32, 34</td>
<td>100 x 7</td>
<td></td>
<td>25±11 (14–39)**</td>
<td></td>
</tr>
</tbody>
</table>

Five C57BL/6 mice per group were administered i.v. with polypeptides at the indicated days before or after surgical excision of primary tumors (on day 21). Mice were killed 2 weeks after tumor excision, and lung tumor colonies were counted. *P<0.005, **P<0.001.
radiolabeled polypeptide can be decomposed into small molecular weight fragments by the treatment with fresh mouse serum (38). These results suggest that the enhancement of the peptide-mediated inhibition of experimental and spontaneous tumor metastasis is related to the relatively slow clearance rate in the circulation.

VI-2. Tumor cell adhesion to ECM and secondary structure of poly(RGD)
Poly(RGD) or fibronectin promoted the adhesion of B16-BL6 melanoma cells when they were immobilized on the surface of tissue culture wells, while tumor cell adhesion to fibronectin or poly(RGD) substrates was specifically inhibited by the addition of RGDS or poly(RGD) in a concentration-dependent manner (90). The anti-adhesive activity of poly(RGD) was more effective than that of RGD-containing oligopeptide. In contrast, random poly(R,G,D) did not affect tumor cell adhesion to fibronectin substrate. Similarly, poly(RGD) significantly inhibited the binding of radiolabeled fibronectin to tumor cell monolayers compared with RGD oligopeptide on a molar concentration basis (95, 96). These results indicate that poly(RGD) may preferentially bind to the RGD-dependent integrin receptor on the cell surface in comparison with monomeric peptide, thus reflecting the ability of poly(RGD) to interfere with tumor cell adhesion to host cells and ECM components including fibronectin.

Structurally, fibronectin is made up of a nonglobular, asymmetric rod-like molecule, with a few organized structures such as α-helices or β-structures. Pierschbacher and Ruoslahti (13) have reported that the region containing the RGDSP sequence of fibronectin may be a β-turn and is thus available to interact with cell surface receptors (integrin). The secondary structure of poly(RGD) was predicted to be a β-turn from its circular dichroism spectrum more frequently than that of RGD oligopeptide and random poly(R,G,D) and by the method of Chou and Fasman based on its primary structures (95). The inhibition of cell adhesion mediated by poly(RGD) may be in part due to its potent binding capacity to fibronectin receptors (integrins) on the cell surface, probably through its conformational properties.

VI-3. Inhibition of release of tumor cells from the primary tumor site
As shown in Table 4, single intralesional (intratumoral) administration of poly(RGD) on day 0, 1 or 7 after tumor inoculation caused a marked reduction of colonies of B16-BL6 melanoma in the lung without suppressing the growth of the primary tumor (92, 97). Such an inhibitory effect of intralesional administration of poly(RGD) on lung metastasis may be partly due to the inhibition of tumor-induced angiogenesis (neovascularization), thereby resulting in interference with active migration or release of tumor cells from the primary tumor site, in addition to blockage of the adhesion of metastasising cells to the surrounding tissues.

VI-4. Tumor cell arrest
To investigate the effect of poly(RGD)on the arrest and lodgement of tumor cells in the capillary bed of the chosen organ, we examined the organ retention of 125I-deoxyuridine-labeled B16-BL6 cells (Fig. 5). The coinjection of radiolabeled melanoma cells with poly(RGD) led significantly to a reduced arrest and retention of tumor cells in the lung (target organ) at 4 and 24 hr after the injection.

Table 4. Effect of polypeptides on spontaneous lung metastases by intrafootpad injection of B16-BL6 melanoma cells

<table>
<thead>
<tr>
<th>Administered i.v. with:</th>
<th>Dose (ng/mouse)</th>
<th>Primary tumor size on day 21 (mm ± S.D.)</th>
<th>No. of lung metastases on day 35</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean ± S.D. (Range)</td>
<td></td>
</tr>
<tr>
<td>Untreated (PBS)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poly(RGD)</td>
<td>on day 0</td>
<td>9 ± 2</td>
<td>44 ± 22 (22–80)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poly(RGD)</td>
<td>on day 1</td>
<td>7 ± 3</td>
<td>6 ± 6 (0–14)**</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poly(RGD)</td>
<td>on day 7</td>
<td>8 ± 2</td>
<td>6 ± 6 (0–14)**</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poly(RGD)</td>
<td>on day 14</td>
<td>8 ± 2</td>
<td>6 ± 6 (0–14)**</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated (PBS)</td>
<td></td>
<td>10 ± 3</td>
<td>46 ± 4 (43–51)</td>
</tr>
<tr>
<td>Poly(RGD)</td>
<td>on day 7</td>
<td>10 ± 3</td>
<td>12 ± 6 (9–21)**</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poly(R,G,D)</td>
<td>on day 7</td>
<td>11 ± 3</td>
<td>58 ± 23 (43–84)</td>
</tr>
</tbody>
</table>

Five C57BL/6 mice per group were administered intratumorally (i.t.) with polypeptides at the indicated days after tumor inoculation. Primary tumors were surgically removed on day 21, and mice were killed 2 weeks after tumor excision. **P < 0.001.
Fig. 5. Organ distribution and retention of $^{125}$I-IUdR-labeled B16-BL6 melanoma cells co-injected with poly(RGD) into C57BL/6 mice. $^{125}$I-IUdR-labeled B16-BL6 cells (1530± 1605 c.p.m./2 $\times$ 10$^4$ /mouse) were injected with or without 500 $\mu$g poly(RGD) into the lateral tail vein of C57BL/6 mice. At the indicated times, mice were killed and radioactive elements retained in the organs were measured. Results are the mean c.p.m.±S.D. of three mice per group. *P<0.02, **P<0.001, as compared with the untreated control (PBS) by Student's two-tailed t-test.

However, there were no discernible differences between untreated control and poly(RGD)-injected mice in the arrest of labeled tumor cells in the liver, spleen, kidneys and blood after the injection. The inhibition of lung colonization by poly(RGD) may therefore depend on the decrease in the arrest of tumor cells in the lung as a result of the inhibition of the adhesive interaction.

VI-5. Adhesive interaction of tumor cells in the circulation

Metastasising tumor cells, once in the circulation, encounter various host cells and/or host components. Among them, platelets are known to play an important role in the regulation of tumor metastasis (8, 98). Many different types of tumor cells have been seen to elicit the activation and aggregation of platelets in vitro (27). These properties have been correlated with the metastatic potential of tumor cells. Various inhibitors of platelet functions have also been reported to retard tumor metastasis in some tumor models. Poly(RGD) completely inhibited tumor-elicited platelet aggregation in vitro, but the random polypeptide poly(R,G,D) did not (Fig. 6) (37, 38, 91). Poly(RGD) and its analogues blocked platelet-
aggregating properties induced by ADP, collagen or thrombin stimuli more effectively than monomeric peptides (37).

As described in VI-2, RGD-containing peptides including poly(RGD), when added freely in solution, inhibited tumor cell adhesion to fibronectin substrate, as compared with unrelated peptides. At the same time, tumor cell adhesion to fibronectin was found to be enhanced remarkably in the presence of platelets (Fig. 7) (38). It has been reported that the mechanism by which platelets enhance tumor cell adhesion to the subendothelial matrix is mediated by surface contact between tumor cells and platelets and depends on a platelet's membrane component and cytoskeleton (99). During the metastatic process, platelet aggregation induced by tumor cells may provide additional means of adhesion through the interaction of platelets with adhesion proteins such as fibrinogen and fibronectin, as well as the opportunity to consolidate the adhesion through a thrombotic formation caused by platelet activation and the deposition of fibrin. Poly(RGD) markedly inhibited the platelet-enhanced adhesion of melanoma cells to fibronectin substrate (Fig. 7) (37, 38). RGD-containing peptides have been shown to inhibit the interaction of adhesive proteins such as fibrinogen, fibronectin and von Willebrand factor with a platelet membrane, presumably by means of the glycoprotein complex IIb/IIa (3-integrin) that serves as a receptor on platelets for such adhesive proteins (36). A IIb/IIa-like glycoprotein identified on endothelial cells may also serve as a matrix receptor (100). Thus, the inhibition of platelet aggregation and platelet-enhanced tumor cell adhesion to fibronectin by poly(RGD) strongly implicates that the RGD sequence competitively blocks

![Fig. 7. Effect of platelets and/or poly(RGD) on the adhesion of B16-BL6 cells to fibronectin-coated substrates. 125I-IUdR-labeled B16-BL6 cells (2 x 10⁴/mouse) were added to the wells coated with 5 µg/ml fibronectin in the presence or absence of platelets (5 x 10⁵/µl) and/or 100 µg/ml poly(RGD) and incubated at 37°C for 10 min. PRP, platelet rich plasma; PPP, platelet poor plasma. (Reprinted from ref. 38 with permission)](image)

![Fig. 8. Effect of poly(RGD) or various antibodies on the homotypic aggregation of B16-BL6 melanoma cells in vitro. B16-BL6 (2 x 10⁴/0.25 ml) cells were incubated at 37°C for 1 hr with or without polypeptide or antibodies. After incubation, the cells were fixed with 10% glutaraldehyde in PBS. The degree of cell aggregation was calculated by visual counting and expressed as the aggregation index (N₁h/N₀h)±S.D. A, cell aggregates after 1 hr-incubation; B, a single cell suspension before incubation. *P<0.001, as compared with the untreated control by Student's two-tailed t-test.)](image)
Table 5. Effect of anti-asialoGM1 serum, 2-chloroadenosine or carrageenan on poly(RGD)-mediated inhibition of lung metastases of melanoma cells

<table>
<thead>
<tr>
<th>Treatment of mice</th>
<th>Poly(RGD)</th>
<th>No. of lung metastases on day 14</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100 μg</td>
<td>Mean ± S.D. (Range)</td>
<td></td>
</tr>
<tr>
<td>Untreated (PBS)</td>
<td>−</td>
<td>119 ± 16 (101 - 138)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>57 ± 7 (48 - 65)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Anti-asialoGM1, i.v.</td>
<td>(20 μg)</td>
<td>144 ± 17 (124 - 169)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>59 ± 14 (44 - 76)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2-chloroadenosine, i.v.</td>
<td>(50 μg)</td>
<td>345 ± 28 (302 - 370)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>21 ± 5 (18 - 27)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Carrageenan, i.p.</td>
<td>−</td>
<td>332 ± 20 (304 - 352)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>39 ± 14 (25 - 58)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

B16-BL6 cells (5 × 10⁴) were injected i.v. with or without poly(RGD) into untreated mice or the mice pretreated 24 h earlier with the indicated agents or antiserum. Lung tumor colonies were determined 14 days after tumor inoculation. *, by Student’s two-tailed t-test.

(Reprinted from ref. 38 with permission)

any interaction between tumor cells, tumor-stimulated platelets (presumably the IIb/IIIa complex) and such adhesive proteins as fibrinogen and fibronectin. However, poly(RGD) had no adverse effects on hemostasis as measured by bleeding time.

On the other hand, tumor-tumor interaction may also form homotypic cell clumps and aggregations in the circulation that can subsequently be arrested and extravasated. Poly(RGD) and anti-LFA-1 antibody did not affect the homotypic aggregation of melanoma cells 1 hr after its co-incubation with tumor cells, while mAbs against N-cadherin or Galα1-4GlcNAcβ1-4 markedly inhibited the aggregation of tumor cells (Fig. 8).

We speculate that some circulating tumor cells are broken by physical blood shear or killed by the immune effectors such as NK cells or macrophages in the circulation during metastasis. We therefore investigated whether or not poly(RGD) can stimulate these immune cells to induce the inhibition of tumor metastasis. Anti-asialo GM1 serum can selectively eliminate NK cells (101), and 2-chloroadenosine (102) and carrageenan were macrophage toxic substances. The pretreatment with anti-asialo GM1 serum, 2-chloroadenosine or carrageenan 24 hr before i.v. injection of tumor cells enhanced the frequency of experimental lung metastasis as compared with the frequency found among untreated normal mice (Table 5). The coinjection with poly(RGD) led to a significant reduction of lung tumor colonies in both untreated and treated mice. Since poly(RGD) was still active in inhibiting metastasis even when the contributions of NK cells or macrophages were removed from this system, its inhibitory mechanism is likely to be unrelated to the stimulation and activation of these cells (38).

V1-6. Tumor invasion to ECM and basement membrane

Although the recognition of endothelial cell surface components is important for the initial attachment of tumor cells to the capillary wall, the adhesion of tumor cells to the ECM and to the basement membrane underlying endothelial cells is equally of significance. The reason for this is that the ECM becomes exposed soon after tumor cells interact with endothelial cells, and tumor cells adhere and invade preferentially to ECM components. The tumor invasion through the ECM and the basement membrane is a complex process involving cell adhesion,
motility (migration), and secretion of various types of degradative enzymes (Fig. 1). Poly(RGD) failed to inhibit tumor cell attachment to endothelium, but blocked the adhesion to fibronectin substrates more potently than RGD-containing oligopeptide by an RGD-dependent mechanism (38, 90, 92). In addition, poly(RGD) significantly inhibited the invasion of tumor cells into reconstituted basement membrane Matrigel (Fig. 9) and haptotactic and chemotactic migration to fibronectin in vitro more effectively than the original RGD-containing oligopeptides (37).

Liotta et al. (103) have reported a correlation between spontaneous metastatic potential and the type IV collagen-degradative activity of tumor cells. Poly(RGD) showed no inhibitory effect on the degradation of [3H]-labeled type IV collagen by tumor cells. These results suggest that the inhibition of tumor cell invasion into the basement membrane by poly(RGD) is mainly due to the prevention of tumor cell adhesion and migration and not to the inhibition of the degradation of ECM and basement membrane by MMP derived from tumor cells.

VI-7. Tumor-induced angiogenesis

Most solid tumors are capable of continuously inducing the formation of new capillary blood vessels from the host vascular bed (angiogenesis) for the supply of nutrients and oxygen and for the removal of their wastes (79, 80, 104). Angiogenesis also allows tumor cells to metastasize from the primary or secondary sites to distant organs (79). Therefore, inhibition of tumor neovascularization may become an important approach for preventing tumor growth and metastasis.

Poly(RGD) significantly reduced the number of capillary vessels oriented towards the tumor mass (angiogenic response) without affecting tumor cell growth when it was coinjected with tumor cells or separately injected i.t. or i.v. on day 1 or 3 after tumor inoculation (Fig. 10) (97). This inhibitory effect of poly(RGD) was dose-dependent. However, RGDS monomer peptide did not show any effect at the dose of 100 μg. Endothelial cell migration toward tumor masses following the formation of capillary sprouts is an essential component in the process of tumor neovascularization. Poly(RGD) also inhibited the

![Fig. 10](image-url)
haptotactic migration of endothelial cells along a gradient of subtratum-immobilized fibronectin but not laminin. Tumor-conditioned medium by itself did not act as a chemoattractant, but promoted the endothelial cell migration to immobilized fibronectin or laminin. Poly-(RGD) inhibited the enhanced cell migration to fibronectin in response to tumor-derived conditioned medium probably including tumor angiogenic factor (97). The inhibitory mechanism by poly(RGD) may therefore depend on interference in the interaction between endothelial cells (mediated by RGD-dependent integrins) and fibronectin. These results suggest that the inhibition of tumor-induced angiogenesis by poly(RGD) partly contributes to the inhibition of spontaneous tumor metastasis, i.e., formation of the established tumor colonies at secondary sites.

V. Other attempts to inhibit tumor metastasis

Despite the importance of the RGDS/integrin interaction in fibronectin-mediated cellular responses, there is evidence for the involvement of additional active determinants within fibronectin and other surface macromolecules. McCarthy et al. (88) have reported that fibronectin contains RGDS-dependent or independent adhesion-promoting activities. A 33-kD heparin-binding fragment, which originates from the carboxyl-terminal end of the fibronectin A chain (Fig. 3), was active at promoting the RGDS-independent adhesion and the spreading of murine tumor cells but not the cell motility. This fragment was able to inhibit experimental metastasis after coinjection with tumor cells (65, 88). Straus et al. (105) found that the migration-enhancing activity was in the fragment containing the cell-binding and heparin-binding (i.e., Hep-2) domains but not other domains. Others have observed similar results using a variety of normal and transformed cell types. CS1 peptide has been shown to be present within the type III homology connecting segment (IICHS) of the 33-kD heparin-binding fragment of fibronectin (Fig. 3) and to promote cell adhesion through an integrin (α5β1)-dependent mechanism (86). Further evidence shows that another cell adhesion domain is located on the amino-terminal side of the RGDS sequence (106).

![Graph and images showing liver metastasis by i.v. injection of L5178Y-ML25 lymphoma cells. Five CDF1 mice per group were inoculated i.v. with L5178Y-ML25 (4 × 10⁴) with or without recombinant fibronectin polypeptides. Mice were killed 16 days after tumor inoculation. The photograph represents liver and spleen metastases in mice given i.v. L5178Y-ML25 cells admixed with or without 100 μg of CH-271 polypeptide. (Reprinted from ref. 107 with permission)
V-1. Recombinant fibronectin polypeptides and their fusion polypeptides

We therefore prepared recombinant fibronectin polypeptides containing the cell-binding domain (C-274) or the heparin-binding domain (H-271), as well as their fusion polypeptide (CH-271) (Fig. 3) and investigated their antimitastatic effects and biological characterizations. The CH-271 fusion polypeptide containing C-274 and H-271 domains was much more effective than H-271, C-274 or their mixture (C-274 + H-271 at a similar molar ratio to CH-271) for inhibiting liver metastases of L5178Y-ML25 T-lymphoma (Fig. 11) and RAW117-H10 B-lymphoma and lung metastasis of B16-BL6 melanoma when co-injected with tumor cells. CH-271 also prolonged the survival of mice by either coinjection or separate injection after tumor inoculation (87, 107, 108). CH-271 inhibits liver metastasis of lymphoid tumor such as L5178Y-ML25 cells by a heparin-binding domain-dependent mechanism (Fig. 11), but suppresses lung metastasis of melanoma or colon carcinoma, depending on the RGD sequence in the cell-binding domain of CH-271 (87). Also the pretreatment of CH-271 polypeptide with IST-1 mAb against heparin-binding domain, rather than mAb against cell-binding domain, abolished the suppression of the CH-271-mediated antimitastatic property using L5178Y-ML25 cells. These findings indicate that the fusion of H-271 with C-274; i.e., CH-271 facilitates the inhibitory effect on tumor metastasis. The interaction between the tumor cell surface and the heparin-binding domain of CH-271, in addition to that between cell surface RGDS-dependent integrins and the cell-binding domain, may lead to the augmentation of such effects perhaps by altering binding affinity.

The reason why CH-271 was more effective in inhibiting tumor metastasis than C-274 or H-271 can not be explained in terms of a difference in the stability in the circulation or in the molecular size of the polypeptides because there was no discernible difference among the polypeptides in the clearance of the radiolabeled polypeptide from the circulation after i.v. injection (107). The CH-271-mediated inhibitory effect is unlikely to be directly or indirectly related to the stimulation and activation of immune cells such as NK cells and macrophages or the cytotoxicity toward tumor cells (107). The co-injection of tumor cells with CH-271 resulted in a significant decrease in the arrest and retention of radiolabeled L5178Y-ML25 cells in the liver, as compared with the finding using a mixture of C-274 and H-271. Therefore, the mechanism responsible for the inhibition of tumor metastasis by CH-271 is partly due to interference with cellular functions such as adhesiveness, motility and invasiveness (87, 107-110) and is basically similar to that of poly(RGD).

Tumor arrest initiated by tumor cell-endothelial cell contact followed by extravasation is an important step in hematogenous metastasis. The adhesion of tumor cells to host endothelial cells in target organs for metastasis formation appears to involve multiple molecular interactions. Pretreatment of the hepatic sinusoidal endothelial (HSE) cell monolayer with liver-metastatic L5178Y-ML25 lymphoma cells or their conditioned medium for 4 to 6 hr resulted in the enhancement of lymphoma cell adhesion to an HSE cell monolayer (47). The increased adhesiveness was completely abolished by pre-incubation of the conditioned medium with anti IL-1,5 mAb or by addition of synthetic SLEx as a ligand for ELAM-1/E-selectin or anti ELAM-1 antibody (47) (Fig. 12). In contrast, pretreatment of an HSE cell monolayer with anti-VCAM-1 or anti-ICAM-1 antibodies or pretreatment of lymphoma cells with anti-LFA-1 antibody did not affect the inhibition of

![Fig. 12. Inhibitory effect of synthetic SLEx, anti VCAM-1 or anti ELAM-1 mAbs on the adhesion of L5178Y-ML25 lymphoma cells to hepatic sinusoidal endothelial (HSE) cell monolayers. Lymphoma cells were added to tumor conditioned medium-stimulated HSE cell monolayers, which had been preincubated with synthetic SLEx, anti VCAM-1 mAb (upper panel) or anti ELAM-1 (21KC10) mAb (lower panel) for 30 min at room temperature, and incubated for 20 min with shaking. Remaining attached cells were counted under a microscope. *P<0.01. (Reprinted from ref. 47 with permission)
enhanced tumor cell adhesion to the HSE monolayer. These results indicate that tumor cell interaction with stimulated HSE cells is primarily mediated by ELAM-1 molecules on the HSE cell surface. This suggests that the initial attachment of a few circulating tumor cells to the endothelium and tumor-derived IL-1β can actually lead to the activation of the endothelial cells and subsequently to the enhancement of tumor cell adhesion to the activated cell monolayers. ELAM-1-mediated enhancement of tumor cell adhesion to the HSE monolayer was also inhibited in a concentration-dependent manner by CH-271 fusion polypeptide or a sulfated chitin derivative that can bind to the heparin-binding domain of CH-271. These findings indicate that the heparin-binding domain of CH-271 is responsible for the inhibition of ELAM-1-mediated tumor cell adhesion to the activated HSE cell monolayers. CH-271 also inhibited the invasion of lymphoma cells into reconstituted basement membrane Matrigel in a concentration-dependent manner.

Interestingly, CH-271 inhibited the adhesion of melanoma cells to substrates precoated with laminin as well as fibronectin, whereas C-274 inhibited cell adhesion to fibronectin by an RGDS-dependent mechanism but not to laminin (Fig. 13). Tumor cell adhesion to laminin was inhibited by the addition of or the treatment of laminin-substrate with heparin or heparan sulfate, which were able to bind laminin. These findings suggest that heparin-like molecules on the cell surface (presumably heparan sulfate proteoglycan, HSPG) are laminin receptors and that the interaction of the heparin-binding domain of CH-271 with the cell surface leads to the inhibition of cell adhesion to laminin (109). Similarly, haptotactic migration of tumor cells to CH-271- or fibronectin-coated substrates was inhibited by the addition of heparin or IST-1 mAb against heparin-binding domain of fibronectin (109, 110). Thus the inhibitory effect can be attributed to the adhesive interaction between tumor cells and the heparin-binding domain in CH-271 (Fig. 14).

To study the regulatory role of the heparin-binding domain in CH-271 fusion peptide on the spreading of HT-1080 fibrosarcoma cells, CH-271 variant peptides lacking various type III modules within the heparin-binding domain were used (111). CH-271 and its variants including the III13 module of heparin-binding domain promoted cell spreading and induced the accumulation of vinculin (a well-characterized cytoplasmic protein) at the focal adhesion of spread cells much more effectively than the variants lacking the III13 module and C-274 without the heparin-binding domain (111) (Fig. 15). Similarly, the

![Fig. 13. Effect of fibronectin and its domain polypeptides on tumor cell adhesion to laminin or fibronectin. Radiolabeled B16-BL6 cells (2 x 10⁶), which had been pretreated with or without fibronectin or its domain polypeptides for 30 min and then washed twice, were added to wells precoated with 10 µg/ml laminin or fibronectin. After a 30-min incubation, nonadherent tumor cells were washed away and the attached cells were counted.](image)

![Fig. 14. Proposed mechanism for inhibition of adhesive interaction between tumor cells and either laminin or fibronectin by CH-271 fusion polypeptide.](image)
variants possessing III13 and/or III14 modules were more active at promoting chemotactic migration than the variants lacking these modules, but the promoting activity of cell migration was abolished by the treatment of HT-1080 cells with heparitinase. These results suggest that spread and migration responses of HT-1080 cells onto the fusion polypeptides require the adjacent coexistence of cell- and heparin-binding domains and are mediated by the interactions between cell surface HSPG and the heparin-binding domain (particularly III13 or III14 modules), in concert with the interaction between cell surface integrins and the cell-binding domain (Fig. 14).

To enhance further the CH-271-mediated antimetastatic effect, we tested a combined therapy with anti-cell adhesive CH-271 peptide and anticancer drugs. Combined treatments with CH-271 and either doxorubicin (DOX) or mitomycin (MMC) significantly inhibited liver and lung metastasis of lymphoma or melanoma cells, respectively, as compared with either treatment alone or the untreated control (112). Combination of CH-271 and DOX substantially increased the survival time of mice injected i.v. with lymphoma cells. These results indicate that the combination of anti-cell adhesive CH-271 and anticancer drugs, i.e., anti-adhesion therapy and chemotherapy, clearly produced an enhancement of the inhibitory effect on tumor metastasis. The combination of CH-271 with DOX provided a more effective inhibition of tumor invasion into Matrigel than did either treatment alone. The inhibition of tumor invasion by CH-271 may be due in part to the anti-cell adhesive property and not affect the growth inhibition of tumor cells, whereas the anti-invasive effect of DOX was established to have resulted from the growth inhibition of tumor cells (112). Thus the antimitastatic effect of CH-271 and DOX in combination may be associated with the operation of distinct inhibitory mechanisms. This further illustrates that anti-adhesion therapy in combination with chemotherapy may be potentially useful for the prevention of cancer metastasis and invasion.

V-2. Sulfated carboxymethyl chitin

Heparin is a structurally heterogenous sulfated glyco-
saminoglycan composed of repeating units of \( N \)-acetyl glucosamine and uronic acid (glucuronic acid or iduronic acid) and is known to exhibit a variety of biological properties such as inhibition of blood coagulation by binding to antithrombin III (113), potentiation of angiogenesis (114), interaction with fibroblast growth factors, inhibition of delayed-type hypersensitivity and modulation of cell growth. Interestingly, heparin can bind to characteristic sequences in the domains of adhesion molecules including those of fibronectin, laminin, and type IV collagen, which serve as a substrate in vitro to promote the adhesion, spreading and migration of tumor cells, and it can inhibit experimental lung metastases. Several attempts have been made to inhibit tumor metastasis experimentally by using heparin and its related compounds. Tsubura et al. (115) have demonstrated that sulfated polysaccharides inhibited blood-borne metastasis by interfering with a step in the coagulation pathway such as the formation of tumor emboli caused by platelet aggregation at the early stage of tumor lodgment. On the other hand, chemically modified heparin without anticoagulant properties has been shown to successfully reduce the number of lung metastasis of melanoma cells and to inhibit the heparanase activity of murine metastatic melanoma cells (116).

Chitin, an homogenous structural polysaccharide composed of \( N \)-acetyl glucosamine, is widely distributed in nature and has been reported to have medicinal and pharmaceutical applications (117). We have found that 6-\( O \)-sulfated carboxymethyl chitin (SCM-chitin), which showed much lower levels of anticoagulant and antiplatelet activities than heparin and can bind to the heparin-binding domain (H-271) of fibronectin (118), potently inhibited spontaneous and experimental lung metastases in mice as well as tumor cell adhesion, migration and invasion in vitro (118–121). In contrast, carboxymethyl chitin (CM-chitin) and SCM-chitosan did not show any activities. The binding of \( ^3 \text{H} \)-heparin to immobilized laminin was also inhibited competitively by the addition of SCM-chitin. These findings suggest that such inhibitory effects are due to the specific binding of SCM-chitin as well as heparin to ECM components.

Degradation of heparan sulfate by heparanase was inhibited by SCM-chitin and heparin, while SCM-chitin inhibited type IV collagenolytic activity of tumor cells more potently than heparin (Fig. 16) (120). Administration of

![Graph](image-url)
SCM-chitin inhibited lung metastasis even in mice that had been pretreated with anti-asialoGM1 serum or carageenan to eliminate NK cells or macrophages (119), and it caused a marked decrease of the number of vessels toward the tumor mass (angiogenic response) without directly affecting the tumor cell growth (121). These results indicate that SCM-chitin-mediated inhibition of tumor metastasis is distinct from that by heparin; and it may be due to the interference with tumor cell invasion consisting of adhesion, migration and enzymatic degradation of basement membrane, and tumor-induced angiogenesis.

V-3. Other RGD-related compounds

The above study using poly(RGD) suggested that polymerization of the RGD sequence was able to augment the inhibition of tumor metastasis and that the inhibitory effect was partly due to slower clearance and decomposition (38). Other attempts, including cyclization of RGD peptide (89, 94), conjugation of RGD sequence with polymeric carriers or the replacement of glycine in the RGDS sequence with either a different amino acid or compound, have also been made to enhance the anti-metastatic and anti-invasive effects.

Most peptides such as RGD-containing peptides have a very short half-life in the circulation, which may result in a decrease of their therapeutic and biological potential in vivo. The control of drug release in vivo may lead to greater effectiveness in the expression of their biological effects. Conjugation of RGD peptides with various carriers such as polyethylene glycol (PEG), poly(carboxyethylmethacrylamide) [poly(CEMA)], CM-chitin or SCM-chitin (Fig. 3) has been shown to augment the ability of the peptide to inhibit experimental and spontaneous lung metastasis in mice as compared with the original RGD peptide, although there is no discernible differences between the peptides and the carrier-conjugated peptides in the degree of inhibition of tumor cell invasion in vitro (94, 122–124). These results indicate that the enhancement of the antitumor effect by conjugation with polymeric carriers may be associated with sustained release of the peptide from the carrier conjugates, slow clearance from the circulation and delayed decomposition, leading to the prolongation of the peptide action. In the case of the enhanced antitumor effect of SCM-chitin-conjugated RGDS (124), SCM-chitin could also serve as a carrier molecule to augment the peptide-mediated effect, in addition to its own antitumor properties as described in V-2. On the other hand, RGD analogues were incorporated into liposomes to stabilize the peptides, to prolong their circulation time and consequently to enhance the antitumor efficacy. Liposomal RGD inhibited experimental and spontaneous lung metastasis more effectively than RGD analogues alone (125).

Several studies have indicated that the R and D amino acids in the RGD sequence are indispensable for the inhibition of cell adhesion and spreading to ECM compo-

![Fig. 17. Effect of doxorubicin (DOX) and/or THF(RGDS)3 on experimental lung metastasis produced by i.v. injection of B16-BL6 melanoma cells. Five C57BL/6 mice per group were inoculated i.v. with B16-BL6 melanoma cells (5 x 10⁴) with or without 1000 μg THF(RGDS)3 on day 0 and/or 100 μg DOX on days 5 and 6. Mice were killed 3 weeks after tumor inoculation and then lung photographs were taken.](image-url)
ments such as fibronectin or vitronectin that occurs as a result of amino acid substitution of the RGDS sequence. However, there have been few studies on the inhibitory effect of R-X-DS peptides, in which the G in RGDS is substituted with other amino acids, on tumor metastasis and invasion. Among the R-X-DS peptide analogues, RLDS inhibited tumor metastasis as effectively as the original RGDS peptide (126, 127). Interestingly, RLDS-containing peptide prevented both the laminin- and fibronectin-mediated invasion of tumor cells, whereas RGDS-containing peptide selectively inhibited fibronectin-mediated invasion. RGDS significantly inhibited tumor cell adhesion to RGDS as well as fibronectin substrates, but not to CS1 (containing EILDV sequence) substrate which is a ligand for the α4β1-integrin receptor on the cell surface. In contrast, RLDS inhibited tumor cell adhesion to CS1 as well as RGDS within the fibronectin molecules. Since Mould et al. (128) have suggested that a new recognition sequence, X-D-Y, can be recognized by α4β1-integrin receptor, RLDS may structurally or functionally mimick the active core sequence (EILDV) in the CS1 peptide, i.e., RLDS vs. EILDV. Thus, the effect of RLDS may retain the properties of both RGDS and EILDV.

Synthetic RGDS analogues conjugated with tetrahydrofurantetracarboxylic acid or trimesic acid, i.e., THF(RGDS)_3 or Ar(DRGDS)_3, significantly inhibited experimental and spontaneous tumor metastasis as compared with RGDS or untreated control (127,129). Both analogues with relatively low molecular weight also showed an inhibitory effect on the invasion and migration of tumor cells in vitro. Combined treatment with THF (RGDS)_3 and an anticancer agent DOX caused marked reduction of the number and size of tumor colonies as compared with either treatment alone (Fig. 17) (129). Administration of DOX alone resulted in marked reduction of the number and size of tumor colonies in the lung. In contrast, treatment of THF(RGDS)_3 achieved a significant decrease in the number of tumor colonies, but the size of tumor colonies was almost the same as that of the control. Thus, the antimetastatic effect of DOX was mainly due to the direct inhibition of tumor growth, differing from that of THF(RGDS)_3, while THF(RGDS)_3 inhibited tumor metastasis in vivo and tumor invasion.

![Fig. 18](image_url)
and migration in vitro in an RGDS-dependent manner without direct cytotoxicity.

To investigate the antimitostatic mechanism by Ar(DRGDS)3 in a living animal, we examined cell trafficking after i.v. injection of [2-18F]2-fluoro-2-deoxy-D-glucose-labeled B16-BL6 melanoma cells by using a positron emission tomography (PET) scanner (130). The real-time PET measurement for the first 120 min, started immediately after injection, showed that tumor cell arrest, i.e., accumulation in the target organ (lung), was remarkably inhibited by liposomal SLe\(^a\) as a ligand for selectin, but not by Ar(DRGDS)3 or liposomal Me-SLe\(^a\) which is not recognized by selectins (Fig. 18). In contrast, Ar(DRGDS)3 inhibited the invasion of B16-BL6 cells into reconstituted basement membrane (Matrigel) following the tumor arrest, whereas SLe\(^a\)- or Me-SLe\(^a\)-entrapped liposomes did not affect tumor invasion (130). In the metastatic processes containing tumor cell lodgement and arrest in the target organ followed by extravasation (invasion), SLe\(^a\) inhibited the initial arrest of tumor cells, presumably the tumor-endothelium interaction, while Ar(DRGDS)3 achieved the inhibition of tumor invasion into basement membrane at later steps of the cascade, consequently leading to the inhibition of metastasis. Thus, tumor cell arrest in lungs in the metastatic processes must be precisely and properly controlled by different adhesion molecules at different stages.

To find anti-metastatic RGDS peptide analogues that possess the property of protease resistance, we synthesized partially modified retro- and retro-inverso peptides of RGD, in which the direction of the \(\epsilon\)-arginine residue is reversed and/or the chirality of the amino acid residue is inverted, according to a report by Nishikawa et al. (131). Retro-peptide Rrev-COCH\(_2\)CO-D inhibited lung metastasis produced by i.v. coinjection with B16-BL6 melanoma more potently than did other pseudo peptides or the original RGDS peptide (132). The invasion of melanoma cells into Matrigel in vitro was suppressed by Rrev-COCH\(_2\)CO-D more effectively than by RGDS. The RGDS peptide decomposed when incubated with fresh plasma in vitro, whereas Rrev-COCH\(_2\)CO-D was not affected by the treatment. Thus, the reversion of the Arg-Gly linkage in the RGD sequence results in protease resistance leading to the retardation of the clearance of the peptide in vivo and consequently augments its anti-metastatic and anti-invasive properties. On the other hand, \(^1\)H-NMR analysis of Rrev-COCH\(_2\)CO-D suggests the involvement of hydrogen bonds between amido-protons of arginine and aspartic acid residues and formation of a particular structural conformation (data not shown), although the conformational analysis has yet to be determined in detail. The structural model shows that Rrev-COCH\(_2\)CO-D has structural similarity to the RGD sequence, which can be recognized by cell surface integrins. Therefore, further study is needed to analyze the affinity of the retro-peptide for cell surface receptors, the selective binding of the peptide against integrins and its relationship with certain biological properties.

In addition, replacement of the malonyl moiety of Rrev-COCH\(_2\)CO-D with a carboxyethylene linkage (Rrev-COCH\(_2\)CH\(_2\)-D) achieved more potent inhibition of lung metastasis of melanoma cells than Rrev-COCH\(_2\)CO-D (133). Among the analogs, a \(p\)-xylylenediamine derivative having two Rrev-COCH\(_2\)CH\(_2\)-D moieties, FC-336, showed the most potent inhibitory effect on experimental lung metastasis produced by i.v. co-injection with B16-BL6 melanoma or colon 26 M3.1 cells in a dose-dependent manner. Interestingly, zymography analysis revealed that FC-336 inhibited the degradation of gelatin substrate by MMPs produced by tumor cells, while the RGDS peptide did not affect the enzymatic degradation. These findings indicate that the pseudo-peptides of the RGD sequence,

![Fig. 19. Activation of CD8+ cytotoxic T cells by tumors. When tumors display antigens on their surface, it is usually in the context of peptides bound to MHC class I molecules. This provides T cell antigen-specific receptor signals to CD8+ T cells but not costimulation. Unless CD4+ T cell help is also present, the cytotoxic T cells are inactivated (anergized?) by the tumor. When B7 is introduced into the tumor cells, they acquire a mechanism for costimulating the CD8+ T cell, so that it can make enough IL-2 on its own to become fully activated and expand. Once activated, the cytotoxic T cell can kill tumor cells that are not expressing B7, because costimulation is not required for cytotoxic effector function.](image-url)
V-4. Control of adhesive interaction between tumor and immune cells

Attempts to control tumor metastasis have been extensively carried out using synthetic cell-adhesive peptides including RGD peptide analogues based on the interference of tumor interaction with ECM components and other cells such as platelets and fibroblasts. Considering the adhesive interaction of tumor cells with immune cells such as cytotoxic T cells, macrophages or NK cells in the metastatic cascade, the efficient promotion of such adhesive interactions may lead to the activation of tumor-specific effector cells and induction of anti-tumor immune responses and thus to the inhibition of tumor metastasis.

Anti-tumor immunotherapy by expressing B7 (B7-1/CD80), a natural ligand for T cell costimulatory molecule CD28, has been recently indicated to stimulate CD4+ and CD8+ T cells and thereby to induce the immunity specific for tumor cells, especially, disseminated tumors (Fig. 19). Several investigators have shown that B7 expression by tumors resulted in protection against a subsequent challenge of non-B7-expressing tumor (wild-type) as well as B7-1-expressing tumor (77, 78, 134, 135). It has also been shown that the degree of the effect of B7-1 costimulation on tumor immunity correlates with the tumor immunogenicity (136). Our previous study demonstrated that the transfection of B7-1 or its variant MB7-2 genes into MHC class I+ tumor cells (B16-BL6 or K1735-M2 melanoma) resulted in the remarkable reduction of lung metastasis caused by the i.v. injection into immunocompetent syngeneic mice (137). However, i.v. injection of the transfectants into T cell-deficient nude mice did not affect reduction of lung tumor colonies as compared with parental wild-type tumors, suggesting that such an inhibitory effect was closely associated with T cell-mediated responses. The reduced metastasis of B7+ tumor cells consequently led to the significant prolongation of mouse survival. However, expression of B7 on

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**Fig. 20.** Effect of immunization with irradiated B7-1 transfected tumor cells and FC-336 on spontaneous metastasis of B16-BL6 melanoma cells. Seven C57BL/6 mice per group were inoculated s.c. with 5 x 10^5 viable B16-BL6 cells into the right hind footpad. Subcutaneous immunization with 10^6 of irradiated B7-12-6 or V9 cells was begun on day 23 after tumor inoculation, and the treatment was performed 2 times at a 7-day interval. FC-336 was administered i.v. 5 times at 1-day intervals on days 16 to 24. Surgical excision of primary tumors was carried out on day 19, and mice were killed on day 41 after tumor inoculation. *P<0.05, **P<0.01, ***P<0.001, as compared with the non-immunized control by Student’s two-tailed t-test.
tumor cells did not influence the tumorigenicity in vivo or tumor cell invasion into basement membrane Matrigel in vitro.

We recently found that pre-immunization of X-irradiated B7-1-transfected B16-BL6 cells was an effective tumor vaccine for preventing lung metastasis caused by i.v. injection of B7-1+ parental cells (137). Vaccinations of irradiated B7-1+ tumor cells before or after surgical excision of the s.c. inoculated primary B7-1 tumors effectively inhibited spontaneous lung metastasis. Thus, vaccination of B7-1+ tumor after surgical excision of primary tumors appeared to be a practical strategy to prevent the recurrence of established metastasis.

As mentioned in section V-3, multiple administrations of FC-336 pseudo-peptide after tumor inoculation inhibited spontaneous lung metastasis through the interference of tumor invasion, migration and adhesion (133). Combined treatment of B7-1 transfected tumor vaccine and anti-adhesive therapy with FC-336 led to the augmentation of the anti-metastatic effect in both experimental and spontaneous metastasis models, as compared with either treatment alone (Fig. 20). Thus, B7-1- or FC-336-mediated inhibition of tumor metastasis may be mediated by different mechanisms at various steps of metastasis, based on the regulation (promotion or inhibition) of tumor cell interaction with host cells and components (138).

VI. Conclusion

During the metastatic process, adhesive interaction between tumor cells and host cells and the components must be precisely controlled by the expression, utilization and combination of different adhesion molecules. Several attempts using a number of related compounds derived from cell adhesion molecules have been made to control the mechanisms involved in cell functions such as adhesion, migration and invasion of tumor cells. Polypeptides derived from adhesion molecules, such as the RGD peptide analogues, markedly inhibited tumor metastasis in a model of spontaneous metastasis by interfering with such tumor adhesive interactions, without affecting short-term toxicity to the host. In addition, the combination of the anti-adhesive peptide and anticancer drug, i.e., anti-adhesion therapy combined with chemotherapy, effectively enhanced the inhibitory effect on tumor metastasis and invasion.

On the other hand, expression of the costimulatory molecule B7 on tumor cells resulted in the promotion of adhesive interaction of tumor cells with immune cells and the efficient induction of specific anti-tumor immune responses leading to the inhibition of tumor metastasis. Thus, manipulation (interference or promotion) of the adhesive interaction of metastatic tumor with the host by using compounds derived from cell adhesion molecules or by introduction of the molecules into the cells may provide a new and promising approach for the prevention and control of cancer metastasis, differing from the suppression of metastasis by conventional anti-cancer drugs.

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