National Library of Canada
Acquisitions and Bibliographic Services Branch
395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale du Canada
Direction des acquisitions et des services bibliographiques
395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

## NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

## AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

Canada

# Role of tumor-hepatocyte adhesion in carcinoma cell metastasis to the liver

by

**Jian Wang**

Department of Surgery, Division of Surgical Research

McGill University, Montreal, Quebec, Canada

November, 1995

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of

**Doctor of Philosophy**

ISBN 0-612-12506-8

To the memory of my father,

in the hope that one day cancer and its metastases will become curable.....

# ACKNOWLEDGMENTS

First of all, I would like to acknowledge Dr. Pnina Brodt, my research supervisor. I am greatly indebted for her continued supervision, guidance and personal support throughout this Ph.D. program. Without her encouragement and support this thesis would not have been accomplished.

To my Advisory Committee Chairman Dr. Anne-Liese D. Recklies, Committee Members Dr. Arthur Sullivan and Dr. Wei H. Lai for their useful suggestions on the project and support. To Dr. Julius Gordon for his support, for his assistance with the FACS analysis as well as his interest in my project. To Dr. Orest Blaschuk for his support and his comments on the manuscripts. To Drs. John Sampalis and Sungsub Chio for their help with the statistical analysis of some data.

To Lucia Fallavollita for her friendship, help with some experiments, efficient coordination of the laboratory and assistance, as well as kind translation of thesis abstract. To John Nip for his kindness, assistance and friendship.

To the laboratory family members Li Long, Margaret Durko, Roya Navab and Keguan Chen and neighbours Patricia Guyda, Linda Bazinet, Anne Gordon, Leopoldo Sanchez-Cantu, Mina Hassan-Zahraee as well as other friends in the Donner Building for their support and the memories they provided. To Irene Sydorenko and Brenda Bewick for their administrative help. To Maria Ribeiro, Alberto Rodrigues and Leslie Johnson for their excellent animal care they provided.

To Martin Robert for his helpful discussions and help with some of the techniques used in this study. To John Leander Po for his help with the FACS analysis.

To McGill Graduate Faculty, McGill Medical Faculty and the Research Institute of Royal Victoria Hospital for financial support.

To my family, many thanks to my father for his spirit, to my mother for her love and encouragement, to my brothers for their support, to my father-in-law and mother-in-law for their understanding.

Finally, great thanks to my husband Yibing Chen, for his deep love, understanding, caring, endless support and encouragement as well as his sense of humour, which made my study possible.

# ABSTRACT

Cell adhesions molecules (CAM) have been implicated in the process of cancer metastasis. However, the interactions of metastatic tumor cells with organ parenchymal cells - hepatocytes particularly the relevance of tumor cell - hepatocyte adhesion to liver metastasis is still not fully understood. We have investigated the role of tumor cell-hepatocyte interaction in liver metastasis using liver metastatic subline H-59 of the Lewis lung carcinoma. A monoclonal antibody (MAb) C-11 was produced which was highly specific to liver metastatic H-59 cells. Our *in vitro* studies showed that this MAb and its F(ab)2 fragments could block tumor H-59 cell adhesion to hepatocytes. The molecule detected by MAb C-11 on H-59 cells as well as on hepatocytes was a 64-71 kDa plasma membrane glycoprotein (designated C-11 BP). We found that this molecule contains approximately 43% of N-linked carbohydrates which were not involved in MAb C-11 recognition, but participated in the adhesion process. MAb C-11 could also block the stimulatory effect of hepatocytes on tumor cell proliferation indicating that the adhesion of H-59 cells to hepatocytes may be necessary for tumor cell growth. The *in vivo* role of the C-11 BP was subsequently tested and it was shown that MAb C-11 and its F(ab)2 fragments could significantly reduce the number of hepatic metastases when used to pretreat H-59 cells before intrasplenic injection, or when inoculated directly into the animals by the intraperitoneal and intravenous routes. This inhibitory effect resulted in an increase in the survival time of tumor-inoculated mice. In additional studies, a second molecule namely the membrane-associated endoplasmic reticulum protein ERp-72 was also implicated in the adhesion of H-59 cells to the hepatocytes, however its role *in vivo* remains to be confirmed. Our results suggest that CAM involved in tumor-hepatocyte adhesion play an important role in liver metastasis. Monoclonal antibody against tumor cell surface adhesion molecules may potentially provide an effective therapeutic agent in the clinical management of liver metastases when combined with traditional therapy.

# RÉSUMÉ

Les molécules d'adhérence aux cellules sont impliquées dans le métastase du cancer. Cependant, les intéractions des cellules de tumeur métastatiques cancéreuses avec les organes parenchymals, en particulier la pertinence des cellules de tumeur et l'adhérence des hépatocytes au foie, n'est pas encore compris pleinement. Nous avons étudié le rôle de l'intéraction entre la cellule de tumeur et les hépatocytes dans le métastase du foie en utilisant une ligne H-59 du carcinoma de poumon Lewis. Un acticorps monoclonal (MAb) C-11 a été produit qui était hautement spécifique au foie et aux cellules métastatiques H-59. Notre étude en vitro a demontré que cet MAb et son F(ab)2 fragment pourraient bloquer l'adhérence de la tumeur H-59 aux cellules des hépatocytes. La molécule détectée par MAb C-11 sur les cellules H-59 ainsi que sur les hépatocytes était une fraction 64-71 kDa de la membrane de plasma glycoprotéine (désignée C-11 BP). Nous avons trouvé que cette molécule contient à peu près 43% de N-liés hydrates de carbone qui n'étaient pas impliqués dans le procédé d'adhérence. MAb C-11 pourrait aussi bloquer l'éffet stimulatoire des hépatocytes sur la prolifération des cellules de tumeur indiquant que l'adhérence des cellules H-59 aux hépatocytes peut être nécessaire pour la croissance des cellules de tumeur. Le rôle en vitro du C-11 BP a été etudié ultériorement et il a été démontre que MAb C-11 et les fragments F(ab)2 pourraient considérablement reduire le nombre de métastases quand on donne un traitement où le C-11 BP est inoculé dirèctement dans les souris par des itinéraires intraspléniques, intrapéritoneales et intraveineux. Cet effet prohibitif a résulté dans un accroissement du temps de survie des souris inoculées de tumeur. Dans l'étude supplémentaire, une deuxième molécule, notamment la protéine du réticulum endoplasmique ERp-72, a aussi été impliqué dans l'adhérence des cellules H-59 au hépatocytes, cepandent son rôle en vitro reste à être confirmé. Nos résultats suggèrent que ces molecules d'adhérence impliqués dans l'intéraction de la tumeur et les hépatocyte jouent un rôle important dans les métastases du foie. Les anticorps monoclonals contre les molécules d'adhérences à la surface des cellules de tumeur peuvent potentiellement fournir un traitement éfficace thérapeutique dans la gestion clinique du métastase du foie quand il est combiné avec la thérapie traditionnelle.

# PREFACE

This thesis is written in accordance to the "Thesis Specification" described in "Guidelines for Thesis Preparation" by Faculty of Graduate Studies and Research of McGill University:

> Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of the thesis.
>
> If this option is chosen, **connecting texts that provide logical bridges between the different papers are mandatory.** The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.
>
> The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". **The thesis must include:** A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.
>
> Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.
>
> In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent.** Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make

perfectly clear the responsibilities of all the authors of the co-authored papers.

This thesis therefore includes two published papers included as Chapters II and III of the thesis:

**Chapter II:** Wang, J., Fallavollita, L., and Brodt, P.: Identification of a Mr 64000 plasma membrane glycoprotein mediating adhesion of tumor H-59 cells to hepatocytes. ***Cancer Research.*** 51: 3578-3584, 1991.

This paper is reproduced from Cancer Research by copyright permission of Ame ican Association For Cancer Research, INC. It presents our *in vitro* results.

**Chapter III:** Wang, J., Fallavollita, L., and Brodt, P.: Inhibition of experimental hepatic metastasis by a monoclonal antibody which blocks tumor-hepatocyte interaction. ***Journal of Immunotherapy***. 16: 294-302, 1994.

This paper is reproduced from Journal of Immunotherapy by copyright permission of Raven Press, Medical and Scientific Publishers. It presents our *in vivo* findings.

These two chapters are preceded by a general introduction and an extensive literature review relevant to the subject matter in Chapter I. Additional unpublished findings are presented in Chapter IV. A general summary and conclusions are presented in Chapter V.

The project was designed by Dr. Pnina Brodt. The author is responsible for most of the experiments in these two published papers except for the immunofluorescence assay and some *in vivo* experiments which were performed by Ms. Lucia Fallavollita.

# CONTRIBUTION TO ORIGINAL KNOWLEDGE

A new cell adhesion molecule expressed on tumor cells and hepatocytes and involved in tumor cell - hepatocyte interaction was identified and partially characterized using a monoclonal antibody.

The adhesion molecule identified by the monoclonal antibody was implicated in the growth promoting effect which hepatocytes exert on tumor cell growth.

The monoclonal antibody and its F(ab)2 fragments did not only block the adhesion of the tumor cells to hepatocytes *in vitro*, but also inhibited liver colonization by the tumor cells. This *in vivo* inhibitory effect resulted in an increased survival time for antibody treated tumor - bearing animals.

A second membrane - associated molecule namely the endoplasmic reticulum protein ERp72 was also implicated in the tumor cell - hepatocyte interaction.

Together our data suggest that antibodies directed to adhesion molecules may have potential applications as effective therapeutic agents in the treatment of liver metastases.

# TABLE OF CONTENTS

Acknowledgments ..... I
Abstract ..... II
Résumé ..... III
Preface ..... IV
Contribution to original knowledge ..... VI
Table of contents ..... VII
Abbreviations ..... XII

CHAPTER I: General Introduction (review of the literature) ..... 1
1. Cancer invasion and metastasis ..... 1
1.1. The clinical significance of invasion and metastasis ..... 1
1.2. Mechanisms of invasion and metastases ..... 1
1.2.1. "Seed and soil" and mechanical hypotheses ..... 1
1.2.2. The metastasis cascade ..... 2
1.2.2. a. Detachment and invasion ..... 3
1.2.2. b. Transport and survival in the circulation ..... 3
1.2.2. c. Arrest at new location ..... 3
1.2.2. d. Attachment and extravasation ..... 4
1.2.2. e. Establishment of new lesions ..... 5
1.3. Cellular events in tumor cell invasion and metastasis ..... 5
1.3.1. The role of cell adhesion cascades in leukocyte migration and cancer metastasis ..... 5
1.3.1. a. Integrins ..... 7
1.3.1. b. E-Cadherins ..... 8
1.3.1. c. Immunoglobulin superfamily ..... 10
1.3.1. c. *(a)*. CEA ..... 10
1.3.1. c. *(b)*. ICAM-1 ..... 11
1.3.1. c. *(c)*. NCAM ..... 12
1.3.1. c. *(d)*. VCAM-1 ..... 13
1.3.1. d. CD44 ..... 13
1.3.1. e. E-selectins ..... 15
1.3.2. ECM degrading enzymes in cancer invasion and metastasis ..... 16
1.3.2. a. ECM ..... 16
1.3.2. b. ECM-degrading enzymes ..... 17

1.3.2. b. *(a)*. MMPs ..... 17
1.3.2. b. *(b)*. The cathepsins ..... 19
1.3.3. The role of growth factors in organ-preference of metastasis ..... 21
1.3.3. a. Autocrine growth factors ..... 22
1.3.3. b. Paracrine growth factors ..... 23
1.3.4. Cell surface N-linked (Asn-linked) carbohydrates and metastasis ..... 25
1.3.4. a. Classification of glycoprotein ..... 25
1.3.4. b. Carbohydrates and metastasis in general ..... 25
1.3.4. c. β1-6 linked branching of N-linked oligosaccharides and metastasis ..... 26

*Figure 1. The metastasis cascades* ..... 28

*Table I: Integrins Expression and Cancer Metastasis (some examples)* ..... 29

2. Liver metastasis: Clinical, Anatomical and Biological aspects ..... 30
2.1. Clinical significance of hepatic metastasis ..... 30
2.2. Prognosis and therapeutics of liver metastasis ..... 30
2.2.1. Surgical resection ..... 30
2.2.2. Chemotherapy ..... 31
2.2.2. a. Systemic chemotherapy ..... 31
2.2.2. b. Hepatic arterial infusion chemotherapy ..... 32
2.2.3. Other forms of therapy ..... 32
2.2.4. Experimental therapy ..... 33
2.3. Anatomical aspects of liver metastases ..... 33
2.3.1. Blood supply of the liver ..... 33
2.3.2. Hepatocytes ..... 34
2.3.3. Hepatic sinusoids and cells ..... 34
2.3.3. a. Kupffer cells ..... 35
2.3.3. b. Sinusoidal endothelial cells ..... 36
2.3.3. c. Pit cells ..... 36
2.3.3. d. Fat storing (Ito) cells ..... 37
2.3.4. The space of Disse ..... 37
2.3.5. Extracellular matrix proteins ..... 38
2.3.6. Hepatic acinar units and their zones ..... 38
2.4. Microenvironment of liver and liver metastasis ..... 39

2.4.1. Cancer cell arrest in the liver ..... 39
2.4.2. Tumor - endothelial cell interaction ..... 40
2.4.3. Tumor cell interaction with the ECM ..... 42
2.4.4. Tumor cell interaction with the hepatocytes ..... 43
2.4.5. Paracrine growth regulation and liver metastasis ..... 45

*Figure 2. The hepatic circulation* ..... 47
*Figure 3. Topography of the hepatocytes, simusoids and the perisinusoidal space of Disse* ..... 48
*Figure 4. The liver acinar unit and the three microcirculatory zones* ..... 49

3. Monoclonal antibodies and cancer therapy ..... 50
3.1. General Introduction ..... 50
3.1.1. Significance of antibodies in cancer therapy ..... 50
3.1.2. HAMA and humanized MAb ..... 50
3.1.3. Functions of antibody fragments ..... 51
3.2. Strategies for therapeutic application of MAb ..... 52
3.2.1. Destruction of tumor cells by immune mechanism ..... 53
3.2.1. a. Antibody Dependent Cell-mediated Cytotoxicity (ADCC) ... 53
3.2.1. b. Complement Mediated Cytotoxicity (CMC) ..... 54
3.2.1. c. ADCC and CMC in experimental and clinical trials ..... 54
3.2.2. Regulation of tumor growth by MAb ..... 55
3.2.2. a. MAb to growth factor receptors ..... 55
3.2.2. b. MAb induction of Apoptosis ..... 57
3.2.3. MAb as carriers of cytotoxic agents ..... 57
3.2.3. a. Radio-immunoconjugates ..... 58
3.2.3. b. Chemo-immunoconjugates ..... 59
3.2.3. c. Immunotoxins ..... 60
3.3. MAb against adhesion molecules ..... 60
3.3.1. MAb to tumor - endothelial cell adhesion receptors ..... 61
3.3.2. MAb to integrins involved in cell - ECM interactions ..... 62
3.3.3. MAb directed to CD44 ..... 63
3.3.4. MAb mediated blocking of other cell - substratum interactions ..... 65
3.3.5. Conclusion ..... 66

4. Lewis lung carcinoma (3LL) and the sublines ..... 67

4.1. Lewis lung carcinoma: Origin and metastatic properties ..... 67
4.2. The sublines H-59 and M-27 of 3LL ..... 67
4.3. Other liver metastasis - associated properties of H-59 cells ..... 69
4.3.1. Adhesion of H-59 cells to ECM proteins ..... 69
4.3.2. H-59 cells secrete BM degrading enzymes ..... 70
4.3.3. E-selectin mediates adhesion of H-59 cells to liver sinusoidal endothelial cells ..... 70
4.3.4. H-59 cells express higher levels of ganglioside GM2 ..... 71
4.3.5. Other carbohydrate moieties on H-59 cells ..... 72
4.3.6. H-59 cells express IGF-1 receptors ..... 72
4.3.7. Conclusion ..... 73

*Figure 5. Lewis lung carcinoma and sublines* ..... 74

5. Rationale and objectives of this study ..... 75

CHAPTER II: Identification of an Mr 64,000 plasma membrane glycoprotein mediating adhesion of tumor H-59 cells to hepatocytes. *In vitro* event ..... 76

CHAPTER III: Inhibition of experimental hepatic metastasis by a monoclonal antibody which blocks tumor-hepatocytes interaction. *In vivo* event ..... 85

CHAPTER IV: ERp72, its expression and possible role in H-59 liver colonization - additional unpublished findings ..... 96
Abstract ..... 97
Introduction ..... 98
Materials and Methods ..... 100
Results ..... 103
Discussion ..... 104
Figure 1 ..... 108
Figure 2 ..... 109
Figure 3 ..... 110
Figure 4 ..... 111

CHAPTER V: Summary and Conclusions ..... 112

*Figure 6. Possible mechanisms involved in liver metastases formation by H-59. tumor cells* ..... 121

Bibliography ..... 122

# ABBREVIATIONS

| | |
|---|---|
| ADCC: | antibody-dependent cell-mediated cytotoxicity |
| BM: | basement membrane |
| BSA: | bovine serum albumin |
| CAM: | cell adhesion molecule |
| C-11 BP: | C-11 binding protein |
| CEA: | carcinoembryonic antigen |
| CMC: | complement-mediated cytotoxicity |
| Col: | collagen |
| ECM: | extracellular matrix |
| ELAM: | endothelial leukocyte adhesion molecule |
| EGF: | epidermal growth factor |
| ELISA: | enzyme-linked immunosorbent assay |
| ERp: | endoplasmic reticulum protein |
| Fab: | fragment of antigen binding site domain of immunoglobulin |
| FACS: | fluorescence-activated cell sorting |
| Fc: | fragment of constant region |
| FN: | fibronectin |
| HCC: | human colon carcinoma |
| HUVEC: | human umbilical vein endothelial cells |
| ICAM: | intracellular adhesion molecule |
| IGF-1: | insulin-like growth factor-1 |
| IgSF: | immunoglobulin superfamily |
| IL: | interleukin |
| i.p.: | intraperitoneal |
| i.s.: | intrasplenic |
| i.v.: | intravenous |
| $Le^x$: | lewis x |
| LFA: | leukocyte function associated antigen |
| LN: | laminin |
| LSEC: | liver sinusoidal endothelial cell |
| MAb: | monoclonal antibody |
| MMPS: | matrix metalloproteinases |
| MW: | molecular weight |

| | |
|---|---|
| NCAM: | neural cell adhesion molecule |
| NC: | nitrocellulose |
| NK: | natural killer cells |
| NMS: | normal mouse serum |
| NRS: | normal rabbit serum |
| PDI: | protein disulfide isomerase |
| PM: | plasma membrane |
| PVDF: | polyvinylidene difluoride |
| s.c.: | subcutaneous |
| $SLe^x$: | sialyl lewis x |
| STKM: | sucrose in TKM |
| THV: | terminal hepatic venule |
| TIMPs: | tissue inhibitor of metalloproteinases |
| TGF-α: | transforming growth factor-α |
| TPV: | terminal portal venule |
| uPA: | urokinase plasminogen activator |
| uPAR: | urokinase plasminogen activator receptor |
| VCAM: | vascular cell adhesion molecule |
| VLA: | very late activation antigen |
| VN: | vitronectin |

# CHAPTER I:

## General Introduction (review of the literature)

# Chapter I: General Introduction

# (review of the literature)

## 1. Cancer invasion and metastasis

### 1.1. The clinical significance of invasion and metastasis

Although some advances have been made in recent years in conventional chemotherapies and surgical techniques, metastasis is still the major cause of failure for cancer therapy. In gastrointestinal cancer, metastasis is the single most important determinant of death. Approximately 25% of patients with newly diagnosed colorectal cancers have clinically detectable metastasis at time of diagnosis (McArdle et al., 1990). At least 30-40% of the remaining patients which are diagnosed as clinically free of metastases, actually have non-detectable micrometastases. If a primary tumor is detected early and removed before metastasis occurs, the cancer will be eradicated. On the other hand, if even microscopic metastases or secondary tumors are already present at the time of diagnosis, these metastases will grow and prove fatal (Hughes and Sugarbaker, 1987).

### 1.2. Mechanisms of invasion and metastases

#### 1.2.1. "Seed and soil" and mechanical hypotheses

Clinical observations have shown that metastases from different types of tumors tend to occur in specific target organs, with the most common sites of spread being the lungs, liver, lymph nodes, bone and brain. To explain this phenomenon, two major hypotheses have been proposed. In the nineteenth century, Stephen Paget (Paget, 1889)

proposed the "seed and soil" hypothesis, in which he postulated that different tumors were predisposed to spread to particular tissues, on the basis of the tissue's (soil) ability to support the growth of these tumors (seed). Another theory postulates that hemodynamic factors namely blood and lymph flow (i.e. mechanical-anatomical factors) determine the target organ site of metastases. The number of metastases in an organ is related to the number of cells delivered into it by the blood and the number of cells that are arrested in the capillaries. Thus the first organ encountered by tumor cells would be the site where the highest number of metastatic foci develop.

The evidences suggest that both mechanisms are important as it has been observed that tumor cells can either bypass proximal organs and selectively colonize specific distal organs (Sugarbaker, 1981), or if the tumor is widely spread, the first encountered organ will be the most common site of metastatic colony formation (Tranberg and Bengmark, 1994). This suggests that both hemodynamic factors and the selective growth properties of tumor cells in certain organs influence the distribution of metastases from human tumors.

**1.2.2.** The metastatic cascade

The process of metastasis formation is a complex cascade of events, which include: (*A*) detachment and release of cells from the primary tumors and invasion of tumor cells into surrounding stroma, (*B*) transport of tumor cells into the lymphatics or the vasculature and survival in the circulation, (*C*) arrest at new location, (*D*) attachment and extravasation into the surrounding parenchyma and finally (*E*) establishment of a new growth at the new site (Hill, 1992) (Fig.1, page 28). To successfully develop into a

metastatic deposit, a cancer cell (or group of cells) must successfully complete each stage and survive to proliferate.

**1.2.2. a.** Detachment and invasion: Detachment- the first step of cell invasion and migration, is associated with rapid tumor growth, and a reduction in cell-cell and cell-matrix adhesion due to changes in the expression of normal adhesion receptors. Mechanical stress and increased activity of various proteolytic enzymes also contribute to this process. Following detachment, tumor cells may be seen to invade the stroma, migrate, and enter local blood vassels (intravasation).

**1.2.2. b.** Transport and survival in the circulation: Following arrest in the first capillary bed encountered (e.g. lung or liver capillaries), only 1-10% cells will survived in the circulation, that is, approximately 90%-99% of cells are lost from their initial site apparently as a result of cell death (Hill, 1992). The possible causes for cell death may include mechanical stress in the small blood vessels, poor nutrition, toxicity due to high oxygen levels in the blood and the action of host immune effector cells such as polymorphs, macrophages, or natural killer (NK) cells.

**1.2.2. c.** Arrest at new location: Tumor cell arrest in the microvessels of the target organs may be associated with thrombus formation involving the interaction of the tumor cells with platelets in the circulation (Gasic, 1984). Such thrombi might provide protection for tumor cells against mechanical trauma due to the blood flow and against host cells. Fibrinolytic enzymes such as plasmin which are produced or activated by the tumor cells may cause breakdown of the thrombi releasing the tumor cells.

**1.2.2. d.** Attachment and extravasation: Once tumor cells arrest in the vasculature of a new site, they may attach and invade through the endothelial cell monolayer and the basement-membrane (BM) - like matrix (Liotta, 1986). Tumor cells attach to the endothelial cells via endothelial cell adhesion molecules such as ICAM-1, VCAM-1 or E-selectin which recognize ligands such as LFA-1 (for ICAM-1), VLA-4 (for VCAM-1) or lectin-binding glycoproteins (for E-selectin) expressed on the tumor cells (see 1.3.1. c. e). The attached tumor cells then extend pseudopodia into the endothelial cell junctions or induce endothelial cell retraction, allowing access to the BM, and migration through the extracellular matrix (ECM) (Fig. 1D. page 28). Tumor cell adhesion to the BM is mediated by cell-surface receptors such as integrins which interact with different ECM components including laminin, fibronectin (FN), vitronectin, type IV collagen, and proteoglycans (Nicolson, 1988a). Finally the tumor cells produce various proteolytic enzymes, such as urokinase-type plasminogen activator (uPA), different metalloproteinases and cathepsins which contribute to the digestion of BM proteins (Liotta, 1990, 1992). The secreted enzymes are activated by other proteases such as plasmin, or by environmental conditions such as acidity to initiate their function. Tumor cells (as well as normal cells) can also produce enzyme inhibitors such as plasminogen activator inhibitor (PAI-1 and PAI-2), tissue inhibitors of metalloproteinases (TIMP-1, TIMP-2) and the cathepsin inhibitor stefin A (see 1.3.2). In tumor cells, the balance which is maintained under normal physiological conditions between activated proteinases and their inhibitors is disturbed, leading to cell invasion into the organ parenchyma.

**1.2.2. e.** Establishment of new lesions: The ability of tumor cells to establish new foci in an invaded organ is also related to the growth conditions in that organ including the release by the organ of specific growth factors-the paracrine growth factors. A correlation has been shown between the ability of organ-derived factors to stimulate tumor growth *in vitro*, and preferential metastasis to that organ *in vivo* (Long et al., 1994, 1995). Growth factors such as IGF-1 (Long et al., 1994) and TGF-α (Herlyn et al., 1990) as well as growth modulating molecules such as transferrin (Rossi and Zetter, 1992) have been implicated in organ-selective metastasis of cancer cells.

## 1.3. Cellular events in tumor cell invasion and metastasis

### 1.3.1. The role of cell adhesion cascades in leukocyte migration and cancer metastasis

Cell adhesion molecules have been implicated in numerous physiological processes, including embryonic and neural development (Edelman and Crossin, 1991), wound healing and inflammation (Elices et al., 1994). During the cellular responses to inflammation, leukocyte recirculation, migration and homing is mediated by interactions of leukocytes with endothelial cells and the extracellular matrix through specific cell adhesion molecules (Dianzani and Malavasi, 1995). Five families of major adhesion receptors, including integrins (e.g. LFA-1, VLA-4), cadherins (e.g. E-cadherin), the immunoglobulin supergene family (IgSF, e.g. ICAM-1, ICAM-2 and VCAM-1), selectins (e.g. L-, E-, P-selectin) and mucin - like molecules (e.g. CD34, GlyCAM-1) as well as other molecules such as CD44 have been shown to be involved in leukocyte migration and homing.

Cytokines and chemoattractants can induce expression of some of these adhesion molecules and therefore play a role in the regulation of these cascades.

Leukocyte - endothelial cell interaction normally requires three sequential steps, including: *first*, reversible "rolling" mediated by lectin-carbohydrate interactions involving leukocyte or vascular selectins and their oligosaccharide ligand. This can be seen within minutes after tissue injury as neutrophils begin to interact loosely with the venule walls "rolling" along affected segments. *Second*, activation of rolling leukocytes by specific chemoattractants or through cell contact - mediated signals capable of triggering secondary adhesion molecules whose function is activation dependent, and *third*, interaction between the activated adhesion molecule (e.g. integrin) and its counter-receptor (e.g. IgSF), leading to strong adhesion and arrest of leukocytes at a single location on the vessel wall and the initiation of migration, and finally extravasation through endothelial cells junctions (Butcher, 1991). The IgSF receptor, PECAM-1 expressed on both leukocytes and endothelial cells appears to function during extravasation and binding of $\beta_1$ integrins to ECM proteins is thought to contribute to invasion into underlying tissues (Hynes, 1994).

Cell adhesion molecules also play a role in the processes of cancer invasion and metastasis since cancer metastasis entails a series of detachment and attachment events which are based on different cell-cell and cell-ECM interactions (see 1.2.2.). These cell adhesion molecules act as both positive and negative regulators and thus play a critical role in tumor cell migration and metastasis formation. Several examples of adhesion molecules which are involved in cancer invasion and metastasis are listed below.

1.3.1. a. Integrins

Integrins are heterodimeric glycoproteins, consisting of two noncovalently associated - $\alpha$ and $\beta$ subunits that can combine to form distinct receptors. The integrin subfamilies are defined by a common $\beta$ subunit. The receptor has a large extracellular domain, a transmembrane domain and a cytoplasmic domain (Ruoslahti, 1989) which links the internal cytoskeletal network of the cell with the extracellular environment. The extracellular domain can bind to a range of ligands both on other cells (cell-cell adhesion) and on ECM proteins (cell-substrate adhesion) such as collagen, laminin, fibronectin, vitronectin and thrombospondin.

In the early stages of tumor growth the decrease or loss of integrin expression may promote tumor cell detachment from the primary site. When the cells penetrate the adjacent ECM and blood vessels, increased expression of specific integrins may facilitate tumor interactions with vascular platelets or leukocytes (Rao, 1992) and may mediate tumor cell arrest and extravasation.

A relationship between integrin expression and the metastatic potential of cancer cells has been demonstrated in several tumor systems. Some of these findings are listed in table I (page 29). Several studies have shown that during progression of tumor cells from low to high metastatic potential, the expression of specific integrins on tumor cells may change and this may facilitate interactions of tumor cells with platelets ($\alpha_{IIb}\beta_3$), leukocyte ($\alpha_L\beta_2$), endothelial cells ($\alpha_4\beta_1$) and specific ECM proteins such as collagen ($\alpha_2\beta_1$), laminin ($\alpha_2\beta_1$, $\alpha_6\beta_4$), FN ($\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_3$) and VN ($\alpha_v\beta_3$) (table I). However, evidence has also been presented that integrin expression can be inversely correlated with

the invasive potential of cancer cell lines suggesting that some integrins such as $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$ and $\alpha_5\beta_1$ could also play a negative role in cell invasion and metastasis (table I, page 29).

Integrins may also cooperate with proteolytic enzymes or with other adhesion receptors to mediate metastasis. It was found that metastases-derived melanoma cells which expressed high levels of $\alpha_v\beta_3$ on the cell surface, also expressed elevated levels of the urokinase plasminogen activator receptor (uPAR). Furthermore, $\alpha_v$ antisense phosphorothioate oligonucleotides which suppressed αv synthesis, could also reduce uPAR mRNA levels by approximately 50%. Ligation of the $\alpha_v\beta_3$ receptor on melanoma cells by immobilized antibody resulted in increased expression of uPAR mRNA, indicating that expression of $\alpha_v\beta_3$ on melanoma cells is coordinated with expression of uPAR (Nip et al., 1995). Other studies have shown that the expression type IV collagenase on highly invasive metastatic melanoma cells can be regulated by integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$. Antibodies to $\alpha_v\beta_3$ or anti-$\alpha_5\beta_1$ could increase type IV collagenase expression indicating that signaling via these receptors may regulate melanoma cell invasion (Seftor et al., 1992, 1993). An inverse correlation between integrin and CEA expression was demonstrated in colorectal tumor cells (Nigam et al., 1993), and it was also shown that CEA transfectants had a significantly reduced expression of $\beta_1$ and $\alpha_6$ integrin subunits as well as a down modulation of E-cadherin expression when compared to the parental cell line.

**1.3.1. b.** E-Cadherin

E-Cadherin also known as L-CAM or uvomorulin, is a transmembrane glycoprotein of epithelial cells, which is $Ca^{++}$-dependent homotypic intercellular adhesion

molecule. *In vitro* gene transfection experiments gave rise to the notion that loss of E-cadherin expression might be related to the invasive capacity as well as metastatic potential of tumor cells (Giroldi et al., 1992)

There are a number of studies suggesting that expression of E-cadherin is negatively correlated with the metastatic potential of different cancers. This inverse correlation was observed when biopsies of different cancers such as human prostate carcinoma (Umbas et al., 1992), head and neck squamous-cell carcinoma (Schipper et al., 1991), colorectal carcinoma (Van der Wurff et al., 1994) and renal cell cancer (Katagiri et al., 1995) were tested. E-cadherin has been found to have anti-invasive properties *in vitro* in different cells. For example, when non-metastatic Madin-Darby canine kidney (MDCK) epithelial cells were treated with a MAb to E-cadherin, these cells gained an invasive capability *in vitro* (Behrens et al., 1989). It was also found that E-cadherin is often absent in various de-differentiated human carcinoma cells and that the *in vitro* invasiveness of these cells was abrogated after re-expression of E-cadherin by transfection of E-cadherin cDNA (Frixen et al., 1991). Inhibition of E-cadherin with antibody or deletion of the E-cadherin gene increased tumor cell motility, invasion and metastatic potential and on the other hand, transfection of highly metastatic cells with E-cadherin gave rise to low metastatic potential cells (Giroldi et al., 1992).

The role of E-cadherin in cancer metastasis is still under investigation. Since decreased expression of E-cadherin is considered to elicit detachment of tumor cells from primary lesions- the first stage of metastasis, E-cadherin depletion may be one mechanism of increased malignancy (Schipper et al., 1991). E-cadherin-associated proteins, namely

the catenins, which link the cytoplasmic end of E-cadherin to components of the cytoskeleton may also be involved (Kemler, 1993). An inactive catenin molecule may lead to dysfunction of E-cadherin and thus increase the invasion of tumor cells.

**1.3.1. c.** Immunoglobulin superfamily

The immunoglobulin superfamily (IgSF) consists of a group of molecules that share a common structure, the immunoglobulin-homology unit, which consists of a length of 70 - 110 amino acids forming β-pleated sheets (Hynes, 1994). Among the molecules which belong to this family are major histocompatibility antigens, the T-cell receptor, the platelet-derived growth factor receptor, and colony-stimulating factor-1 receptor as well as cell surface receptors involved in cell adhesion such as neural cell adhesion molecule (NCAM), vascular cell adhesion molecule (VCAM), intercellular cell adhesion molecule (ICAM) and carcinoembryonic antigen (CEA). These molecules play important roles in cell-cell recognition, neural development, leukocyte trafficking, signal transduction, as well as regulation of cancer metastasis.

The members of the IgSF family which have been implicated in tumor metastasis including CEA, ICAM-1, NCAM, and VCAM will be briefly reviewed here.

**1.3.1. c.** ***(a).*** *CEA*: is a 180 kDa oncofetal glycoprotein originally found in the serum of patients with colon carcimoma. *In vitro*, CEA can mediate the $Ca^{++}$-independent, homotypic aggregation of colon carcinoma cells. It was also found that CEA may mediate colon carcinoma cell-matrix binding (Pignatelli et al., 1990a). Other investigators have shown that colorectal carcinoma cells at the invading edge express more CEA mRNA than luminal surface tumor cells, indicating that CEA may function either as an epithelial cell

adhesion protein or as an anti-adhesion protein depending on whether it is cell surface bound or secreted (Jothy et al., 1993). CEA may also enhance the metastatic potential of colorectal carcinoma cells by promoting attachment of tumor cells to the liver Kupffer cells and hepatocytes (Hostetter et al., 1990). Recent findings suggest that CEA may mediate tumor adhesion by downregulating integrin and cadherin expression (Nigam et al., 1993). This would result in increased tumor cell detachment from the primary site in the early stage of dissemination. Recently, Majuri and his colleagues reported that CEA is expressed on the endothelial cells surface suggesting that CEA expressing adenocarcinomas could adhere to endothelial cells via a CEA-CEA interaction, and implicating CEA in tumor cell extravasation and metastasis (Majuri et al., 1994).

**1.3.1. c.** ***(b).*** *ICAM-1:* is expressed on endothelial cells as well as on a variety of other cell types. It mediates a wide range of adhesive interactions together with its ligand, the lymphocyte function-associated-1 antigen (LFA-1) within the immune system (Springer, 1990). ICAM-1 is also involved in malignant diseases. Increased expression of ICAM-1 on melanoma cells was shown to correlate with progression and an increased risk of metastasis (Natali et al., 1990). Furthermore, enhanced metastatic ability of TNF-$\alpha$ - treated malignant melanoma cells is reduced by ICAM antisense oligonucleotides (Miele et al., 1994). ICAM-1 has also been implicated in liver invasion by lymphoma cells as it was found that ICAM-1 expressed on hepatocyte cell surface could interact with LFA-1 on lymphoma cells (Meijne et al., 1994). In contrast, metastatic human renal carcinoma cells were found to express less ICAM-1 than the primary tumor cells as determined by immunohistochemistry (Heicappell et al., 1994).

**1.3.1. c.** ***(c).*** *NCAM:* is a $Ca^{++}$-dependent homotypic and heterotypic cell-cell adhesion molecule that is expressed primarily on cells of neural and mesenchymal origin (Chuong and Edelman, 1984). The function of NCAM in cancer metastasis remains the subject of controversy. It has been shown that NCAM expression is downregulated in migrating cells. As well, loss of NCAM expression in a mouse fibroblast cell line after transformation correlated with loss of contact inhibition of growth (Aoki et al., 1991). This provided evidence for the potential involvement of NCAM downregulation in tumor development. However, other lines of evidences indicated that NCAM expression is positively correlated with the neoplastic potential of some tumors of neural, mesenchymal and neuroendocrine origin including Wilms' tumor and pituitary adenoma cells where it was detected by immunohistochemistry (Jin et al., 1991; Edelman and Crossin, 1991). It has been reported that NCAM expression was significantly increased in aggressive, rapidly metastasizing uveal melanoma primary tumors and in their metastases. This study also found that the NCAM isoforms, lacking the HNK-1 epitope, may be associated with the organ - specific metastatic behavior of uveal melanoma since in liver metastases HNK-1 immunoreactivity was significantly reduced compared to NCAM (Mooy et al., 1995). High NCAM expression on small cell lung cancer was also found to correlate with poor response to chemotherapy and short disease-free and overall survival suggesting a role for their receptor in disease progression (Segawa et al., 1993). Therefore NCAM may play both a negative and positive regulatory function in malignant disease depending on the specific cell type.

**1.3.1 c.** ***(d).*** *VCAM-1:* is expressed on vascular endothelial cells in response to induction by different cytokines and it serves as a receptor for VLA-4 ($\alpha_4\beta_1$ integrin) which could be expressed on leukocytes as well as on malignant cells (Elices et al., 1990). The VCAM-1 - VLA-4 interaction has been found to play a role in several tumor cell-endothelial and /or stromal cell interactions, such as the interaction between melanoma and lung vascular endothelial cells (Okahara, 1994), between renal cell cancer cells and human umbilical vein endothelial cells (HUVEC) (Tomita et al., 1995; Steinbach et al., 1996) and between leukemia / lymphoma cells and human marrow stromal cells (Juneja et al., 1993). MAb to VLA-4 or to VCAM-1 could inhibit TNF-$\alpha$ induced enhancement of the VLA-4 - VCAM-1 interaction in all of these models. The function of VCAM-1 in cancer metastasis may be as an endothelial adhesion receptor for those tumor cells which express the $\alpha_4\beta_1$ integrin, thus facilitating metastatic dissemination. Recent studies have shown however that VLA-4 may act to inhibit melanoma invasion and metastasis suggesting that the contribution of this receptor may be complex (Qian et al., 1994).

**1.3.1. d.** CD44

CD44 is a highly glycosylated cell membrane glycoprotein which is widely distributed on a variety of cell types, including hemopoietic, lymphoid and epithelial cells as well as malignent cells. The major function of CD44 is regulation of lymphocyte adhesion to high endothelial venules (HEV) during lymphocyte migration (Stoolman, 1989) by mediating cell adhesion to hyaluronate and matrix proteins (East and Hart, 1993). CD44 can also interact with osteopontin and may be involved in mediating migration of lymphocytes and monocytes out of the blood-stream into sites of

inflammation. This CD44 - osteopontin interaction may also be regulating metastases formation by neoplastic cells (Weber et al., 1996).

The CD44 protein can be expressed in isoforms resulting from mRNA alternative splicing that allows variant cells to exclude or include specific segments of mRNA in the final transcript, thus forming a family of related proteins from a single gene (East and Hart, 1993). CD44 variants (CD44v) has been implicated in cancer metastasis by studies with animal models which showed that CD44v confers a metastatic phenotype on non-metastasizing rat pancreatic carcinoma cells (Gunthert et al., 1991). The experimental evidence also includes the finding that melanoma clones expressing high levels of CD44 exhibited increased metastatic potential (Birch et al., 1991) and that anti-CD44 MAb which blocked binding of human melanoma cells to hyaluronic acid *in vitro*, also inhibited the growth and metastatic potential of tumor cells *in vivo* (Guo et al., 1994). Overexpression of the standard as well as of specific splice variants of CD44 has been associated with metastasis in a range of human tumors such as colorectal cancer (Takeuchi et al., 1995), gastric adenocarcinomas (Hong et al., 1995), cervical cancer (Kainz et al., 1995) and ovarian cancer (Gardner et al., 1995).

The mechanism underling CD44 mediated upregulation of metastasis is still not fully understood. The evidence showed that the MAb to the metastasis-specific domain of the CD44v molecule could effectively prevent formation of metastases by the transfected cells (Seiter et al., 1993). This MAb interfered with proliferation of metastasizing tumor cells in the draining lymph nodes, probably by directly blocking the interaction with a putative ligand. The possibility that expression of CD44 by tumor cells can lead to signal

transduction mechanisms which are triggered by binding to hyaluronic acid and are metastasis-promoting can not at present be ruled out (Shimizu et al., 1989).

**1.3.1. e.** E-selectin

Selectins are a family of $Ca^{++}$-dependent transmembrane glycoproteins which contain a N-terminal lectin-like domain. These membrane proteins are normally expressed on leukocytes (L-selectin), platelets (P-selectin) and endothelial cells (E-selectin). They are involved in leukocyte migration and lymphocyte homing in the process of inflammation and metastasis (Bevilacqua and Nelson, 1993). Among them, E-selectin (or ELAM-1) was most strongly implicated in cancer metastasis. It can bind to carbohydrate determinants expressed on red blood cells (Phillips et al., 1990) or on the tumor cell surface (Walz et al., 1990).

It has been reported that the carbohydrate antigen $SLe^X$ is highly expressed on colorectal carcinoma cells which are able to bind to cytokine-activated HUVEC ( human umbilical vein endothelial cells) and this binding can be inhibited by an anti E-selectin antibody (Lauri et al., 1991). Recently, Steinbach et al demonstrated that $SLe^X$ and VLA-4 are expressed on renal cell carcinoma (RCC) cell lines. Blocking studies with MAbs directed to $SLe^X$ or VLA-4 on tumor cells and E-selectin or VCAM-1 on endothelial cells markedly inhibited tumor cell adhesion to cytokine - stimulated HUVEC. Their results indicated that these molecules may play an important role in the ability of RCC cells to metastasize (Steinbach et al., 1996).

Carbohydrates other than $SLe^X$ may also be important for E-selectin -mediated adhesion. An antibody to the Lewis Fucosylated Type 1 carbohydrate chain was shown to

block colon carcinoma cell adhesion to IL-1 activated endothelial cells and inhibited tumor cell retention in lungs of IL-1 treated animals suggesting that cell adhesion to ELAM-1 may be mediated by different cell - type specific carbohydrate ligands (Dejana et al., 1992). The sialyl Lewis $^{a}$ ($SLe^{a}$), another ligand for E-selectin was also implicated in cancer metastasis. It was found that the increase in adhesion of pancreatic carcinoma cells to cytokine - ( IL-1β and TNF-α) activated endothelial cells is blocked by anti-E-selectin and anti-$SLe^{a}$ antibody. This suggested that the adhesion was mediated by $SLe^{a}$ on the pancreatic carcinoma and E-selectin on the endothelial cells (Iwai et al., 1993).

E-selectin was also found to be expressed on hepatic sinusoidal endothelial cells stimulated by TNF-α (Brodt et al., submitted). The anti-E-selectin antibody could significantly block the cytokine dependent adhesion of H-59 cells as well as human colorectal carcinoma HM-7 cells to a hepatic sinusoidal endothelial cell line *in vitro*, and could also inhibit liver colonization by H-59 cells *in vivo*. It was found that a mucin-associated carbohydrate structure with fucose and sialic acid in the terminal position was involved in this adhesion. It is most likely that the E-selectin-carbohydrate interactions may play a fundamental role in initiating hepatic arrest of colon carcinoma cells and the formation of liver metastases.

**1.3.2.** ECM degrading enzymes in cancer invasion and metastasis

**1.3.2. a.** ECM

The ECM is the fibrous network of proteins and glycoproteins which separate the different tissue compartments and form BM structures. It is impenetrable to cells and macromolecules under normal physiological conditions. The major constituents of the

ECM are collagen (type IV for epithelial and endothelial BM), elastin, fibronectin, hyaluronic acid, laminin, heparan sulphate proteoglycan, thrombospondin, and vitronectin (Mignatti and Rifkin, 1993). The BM type ECM is generally the first barrier for carcinoma cell invasion following cell detachment from the primary site. Interactions between tumor cells and the ECM also occur at later stages in metastasis including intravasation and extravasation (see 1.2.2. d). Therefore the proteolytic degradation of ECM by different protenases is critical for the metastatic process.

**1.3.2. b.** ECM-degrading enzymes

The enzymes implicated in ECM degradation and invasion include: matrix metalloproteinases (MMPs) such as MMP1, MMP2 and MMP9; serine proteinases such as tissue and urokinase plasminogen activators (tPA, uPA) and plasmin; and cysteine and aspartic lysosomal proteinases such as cathepsin B, L and D. Tumor cells may produce one or more of these proteolytic enzymes or they may activate adjacent stromal cells to produce the enzymes resulting in both instances in degradation of ECM and tumor invasion (Mignatti and Rifkin, 1993).

Numerous studies demonstrated that MMPs (particularly MMP2, MMP9), cathepsins (cathepsin B and L) as well as plasminogen activators are involved in tumor invasion and metastasis. Here, the relevant enzymes involved in liver metastasis of tumor H-59 cells will discussed.

**1.3.2. b.** ***(a).*** MMPs

MMPs are the main physiological mediators of matrix degradation. They are secreted as zymogens and must be activated extracellularly. The major members of this

family are collagenases and stromelysins as well as the recently described matrilysin, metalloelastase and MT-MMP (membrane-type matrix metalloproteinase, MMP-XI) (Ray and Stetler-Stevenson, 1994). They are Zinc-dependent endopeptidases which differ in their substrate specificity and may degrade different components of the ECM. The activity of the metalloproteineases is regulated by a family of natural inhibitors known collectively as tissue inhibitors of metalloproteinases (TIMPs 1-3). Under normal physiological condition, TIMPs maintain a stable level of proteolysis. In addition, the expression and function of the metalloproteinase are regulated by growth factors, oncogenes, and hormones at the transcriptional level (Birkedal-Hansen et al., 1993), whereas plasmin may regulate metalloproteinase activity post-translationally (He et al., 1989). Any interruptions of MMP regulation induced by tumor or other factors will result in excessive degradation of ECM and facilitate invasion and metastasis.

One member of the MMP family, gelatinase A is widely involved in tumor invasion and metastasis. Since it was also identified as a mediator of tumor H-59 invasion, it is discussed in more detail here.

Gelatinase A, also known as MMP-2 or the 72 kDa type IV collagenase, mainly degrades BM collagen type IV. It is regulated by TIMP-2 which can form a complex with either the latent or activated forms of the enzyme (Howard et al., 1991). A variety of studies have indicated that gelatinase A expression is increased in some malignant cells and is positively correlated with invasion and metastasis of various tumors, such as ovarian carcinoma (Autio-Harmainen et al., 1993), colonic adenocarcinoma (Levy et al., 1991) and oral squamous cell carcinoma (Kusukawa et al., 1993). Furthermore, transfection of

the ras oncogene into embryo fibroblasts could induce these cells to secrete high levels of type IV collagenase and exhibit a high incidence of spontaneous metastasis in nude mice (Garbisa et al., 1987). Tumor H-59 cells have been shown to secrete high levels of the 72 kDa gelatinase (Brodt et al., 1992), but produce low levels of endogenous TIMP-2. Invasion of H-59 cells through matrigel could be blocked by adding TIMP-2 to the culture (Navab et al., 1995) suggesting that gelatinase A is involved in H-59 cells invasion through matrigel.

The expression of gelatinase A in tumor cells can be stimulated by ligation of integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ (Seftor et al., 1993), by growth factors such as TGF-β1 (Brown et al., 1990) and by tumor cell derived collagenase-stimulatory factor (TCSF) (Kataoka et al., 1993). The activity of gelatinase A is normally regulated by TIMPs (Howard et al., 1991), but it was also found to be regulated by other factors such as a cell membrane associated activator (Brown et al., 1993) and a membrane-type matrix metalloproteinase (MT-MMP) (Sato et al., 1994), which can activate pro-gelatinase A thereby leading to increased degradation of BM components and enhanced tumor cell invasion.

**1.3.2. b.** ***(b).*** The Cathepsins

Cathepsins are lysosomal endopeptidases found in most cells. They include cathepsins B, H, L, S (cysteine proteinases), cathepsin D (aspartic proteinase) (Twining, 1994). Among them, the cathepsins B, L and D have been implicated in tumor progression. Since tumor H-59 cells also express cathepsin L (high levels) and cathepsin B (low level), their activity will be briefly reviewed.

Cathepsin L (CL):

CL is an endopeptidase which can degrade ECM components, such as collagen, proteoglycan and elastin (Guinec et al., 1993). High levels of CL are present in many human tumors, and are thought to contribute to biochemical processes underlying tumor metastasis. CL expression has been detected in human tumors such as kidney and testicular tumors, non-small cell carcinoma of the lung as well as cancers of the breast, ovary, colon, bladder, prostate and thyroid using slot blot or northern blot analyses (Chauhan et al., 1991). It has also been reported that CL expression was increased in a highly metastatic human breast cancer cell line as compare to a poorly metastatic cell line (Scaddan and Dufresne, 1993). An antibody against CL reduced murine myeloma cell growth (Weber et al., 1994). In ras-transformed, metastatic NIH 3T3 cells a good correlation was found between the levels of ras and CL expression (Chambers et al., 1992).

In some cells, increased expression of both CL and CB was found (Chambers et al., 1992). This is also shown for tumor H-59 cells which express high levels of CL mRNA and lower levels of CB mRNA (Brodt et al., 1992). A cysteine proteinase inhibitor (E-64) could efficiently block invasion of H-59 cells *in vitro* and the formation of liver metastases (Navab et al., 1995). Since cysteine proteinases can be involved in proteolytic cascades where they activate other proenzyme such as pro-urokinase (Schmitt et al., 1992), it is possible that in H-59 cells, CL is required for activation of other enzymes such as gelatinase A (1.3.2. b. *(a)* ), thereby playing an indirect role in tumor invasion and metastasis rather than directly mediating proteolysis of the ECM (Guinec et al., 1993).

Recently, Morris and colleagues reported that mammary carcinoma cells expressing CL showed a greater ability to migrate to form micrometastases after extravasation (Morris et al., 1994), indicating that CL may play a critical role in tumor cell migration and growth in metastasis formation.

Cathepsin B (CB):

CB is the most extensively studied lysosomal cysteine proteinase. Similar to CL, it can also degrade various proteins present in the ECM, such as proteoglycans, FN and collagen (Guinec et al., 1993). Many investigators have demonstrated a correlation between increased expression or activity of CB and increased metastatic capability of animal or human malignancies, such as melanoma (Sloane et al., 1986), human lung adenocarcinoma (Ozeki et al., 1993), human breast carcinoma (Scaddan and Dufresne, 1993) and human colon cancer (Emmert-Buck et al., 1994). Increased expression of CB and decreased activity of the inhibitor were also found in ras-transformed metastatic NIH 3T3 cells (Chambers et al., 1992). The function of CB in tumor invasion and metastasis is similar to CL namely it can directly degrade the ECM (Kobayashi et al., 1993) and in addition, it can also cooperate with other proteinases such as interstitial collagenase (Sires et al., 1995). As discussed above, H-59 cells express both CB and CL as well as high levels of gelatinase A. These enzymes may cooperate in degrading BM or ECM during H-59 metastasis (Brodt et al., 1992).

**1.3.3.** The role of growth factors in organ-preference of metastasis

The growth of cancer cells in particular secondary sites may also be regulated by their ability to respond to local paracrine growth factors as well produce and respond to

autocrine growth factors. Several growth factors have been identified which play a role in autocrine or paracrine growth regulation of tumor cells including carcinoma H-59.

**1.3.3. a.** Autocrine growth factors

Some cancer cells grown *in vivo* and tumor cell lines maintained *in vitro* have the capacity to produce multiple growth factors which can both affect the surrounding host cells and promote tumor cell growth (Nicolson, 1993). Autocrine growth stimulation can occur either through an external autocrine loop namely the growth factor is secreted by the tumor cells and interacts with the receptor extracellularly, or through an internal autocrine loop where the interaction between the growth factor and the receptor occurs in an intracellular compartment (Browder et al., 1989). Several growth factors such as the platelet-derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor (TGF), members of the fibroblast growth factor (FGF) family and insulin-like growth factor-1 (IGF-1) have been implicated in autocrine stimulation of tumor growth.

It has been shown that as tumors progress to more malignant or more metastatic phenotypes, they become less dependent on serum-derived growth factors for their growth *in vitro* (Yee et al., 1988) and begin producing growth factor themselves (Rodeck and Herlun, 1991), suggesting that autocrine growth mechanisms may be involved in metastasis formation. This was also demonstrated by studies with a highly metastatic 3LL subline which was shown to produce high levels of the growth factor TGFβ1 mRNA. The authors postulated that this factor plays a role as a autocrine growth regulator for 3LL metastases (Perrotti et al., 1990).

Gene transfer techniques have been used to test for the involvement of autocrine growth mechanisms in metastases formation. For example, the ras-transfected MCF-7 cells were tumorigenic in the absence of estrogen and secreted elevated levels of TGFα, TGFβ and IGF-1 without a change in their growth factor receptor numbers (Dickson et al., 1987). Conditioned medium from MCF-7 ras cells as well as two of its component growth factors IGF-1 and TGF-α replaced estrogen in stimulating MCF-7 colony formation. Another study showed that NIH-3T3 cells transfected with bFGF gene containing a signal-peptide coding region, acquired the ability to form experimental lung metastases after i.v. inoculation (Egan et al., 1990). This was not seen with cells transfected with bFGF lacking the signal peptide. These evidences support the concept of autocrine induction of the metastatic phenotype.

**1.3.3. b.** Paracrine growth factors

Some cancer cells express growth factor receptor and respond to paracrine growth factors secreted by host cells. The ability of tumor cells to metastasize to specific organ sites has been shown to be regulated by their response to organ-derived growth factors. One line of evidence comes from work by Nicolson and his group. Using murine B16 melanoma cells they found that the lung and ovary-colonizing sublines of the B16 melanoma were responsive *in vitro* to growth-promoting factors released by the target organ tissue such as lung and ovary (Nicolson and Dulski, 1986). It was also shown by Radinsky et al that non-metastatic and highly metastatic human colon carcinoma (HCC) cells could grow at a local site, but growth in the secondary site (i.e. the liver) was seen only with highly metastatic HCC cells (Radinsky, 1993).

Several growth factors which act as paracrine growth factors have been identified such as EGF, PDGF and IGF-1. PDGF was found to be abundantly expressed in lung - conditioned media. It was shown to specifically induce *in vitro* the growth of the highly metastatic subline D122 cells of carcinoma 3LL (Fitzer-Attas et al., 1993). Transferrin (Tf) is an iron transport protein necessary for the *in vitro* growth of many different cell types. It has been identified as a growth stimulator for lung metastatic cells in lung - conditioned medium (Cavanaugh and Nicolson, 1991). It was also found in bone - marrow organ cultures. Purified Tf markedly stimulated the proliferation of prostatic carcinoma cells which are typically metastatic to bone (Rossi and Zetter, 1992). IGF-1 has also been identified as a paracrine growth factor for liver metastatic carcinoma subline H-59 cells (Long et al., 1994, 1995). In the latter study, IGF-1 could be detected in hepatocyte conditioned medium (HCM), and the IGF-1 receptor was found to be expressed on H-59 cells. When HCM was added to serum-deprived cells, IGF-1 could stimulate the proliferation of H-59 cells, but not of M-27 cells which are a 3LL subline metastatic to the lung (Long et al., 1994). Furthermore, transfection of H-59 cells with a plasmid vector expressing IGF-1R cDNA in the antisense orientation, which caused a loss of tumor cell ability to respond to IGF-1 *in vitro*, lead to loss of the metastatic ability *in vivo* (Long et al., 1995) indicating that IGF-1 plays a role as a paracrine growth factor in the regulation of H-59 metastasis to the liver.

### 1.3.4. Cell surface N-linked (Asn-linked) carbohydrates and metastasis

#### 1.3.4. a. Classification of glycoproteins

Glycoproteins are classified according to the nature of linkage between the carbohydrate sidechain and the protein backbone. The major linkages found in membrane glycoproteins are the N-glycosidic linkage between asparagine and N-acetyl-glucosamine (Asn-linked, or N-linked) and the O-glycosidic linkage, between serine or (threonine) and N-acetyl-galactosamine (O-linked) (Schachter et al., 1985). N-Glycosides are more commonly found in mammalian glycoproteins than O-glycosides, but single glycoproteins may have multiple chains, some of which are O-glycosides and some of which are N-glycosides. The synthesis of the oligosaccharide sidechains is catalyzed by a series of enzymes known as glycosyl transferases. N-linked oligosaccharides can be removed enzymatically by treatment with N-Glycosidase F (Tarentino et al., 1985).

#### 1.3.4. b. Carbohydrates and metastasis in general

Glycoconjugates (glycopreteins and glycolipids) are known to be essential components of plasma membranes of all mammalian cells (Lehninger, 1984). The carbohydrate moieties of glycoconjugates are involved in many cell surface phenomena, including antibody and lectin binding and cellular adhesion and recognition (Brandley and Schnaar, 1986). Changes that take place in the surface carbohydrate structures are thought to play an essential role in normal cellular differentiation and to influence the metastatic potential of cancer cells. The study of these changes has been greatly facilitated by the advent of hybridoma technology (Kohler and Milstein, 1975) which has allowed the production of a range of highly specific MAbs for the detection of closely related

carbohydrate structures expressed during embryogenesis, differentiation and oncogenesis (Feizi, 1985).

It has been well documented that cell surface carbohydrate-containing structures and also changes in these structures are involved in tumor cell and host cell interaction during the process of tumor metastasis both in human and in experimental animal models. These changes include increased expression of β,1-6 -branched asparagine-linked oligosaccharides in human colon carcinoma cells (Laferté and Loh, 1992), the increase in expression of the Lewis$^x$ or sialyl- diameric lewis$^x$ antigen on metastatic mouse or human colon carcinoma cells (Kawakami et al., 1994; Matsushita et al., 1991) and increased content of sialic acid in Friend leukemia cells (Benedetto et al., 1989).

Since the adhesion molecule of H-59 cells identified by MAb C-11 is also a N-linked glycoprotein, the N-linked oligosaccharide glycoproteins which were implicated in malignant disease and metastasis will be briefly discussed.

**1.3.4. c.** β,1-6 linked branching of N-linked oligosaccharides and metastasis

One of the more common alterations in transformed malignant cells is the presence of large N-linked oligosaccharides (Smet and Van Beek, 1984). The most discussed is the β,1-6 linked branching of the N-linked oligosaccharide. Dennis and his colleagues have demonstrated that metastatic murine tumor MDAY-D2 cells which bind to the leukoagglutinin (L-PHA) have increased metastatic properties (Dennis et al., 1987). This L-PHA binding requires the β1-6 linked lactosamine antennae. It was found that cell surface glycoprotein, gp130 was a major target of increased β,1-6-linked branching and that the expression of these oligosaccharide structures was directly related to the

metastatic potential of the tumor cells. Induction of increased β,1-6 branching in clones of a nonmetastatic murine mammary carcinoma correlated strongly with acquisition of lung metastatic potential *in vivo*. Treatment of these tumor cells with swainsonine, a non-toxic inhibitor of Asn-linked oligosaccharide synthesis which reduced cell-surface expression of β,1-6 branched oligosaccharides blocked tumor cells invasion *in vitro* and reduced solid tumor growth and metastasis *in vivo* (Dannis, 1986).

Increased β,1-6 branching of N-linked oligosaccharides has also been detected in human malignancies, such as carcinomas of the breast and colon and in melanoma (Fernandes et al., 1991; Dennis, 1991). MAb has been produced to identify the L-PHA-binding glycoprotein on human colon carcinoma cells (Laferté and Loh, 1992). It was found that the glycoproteins detected by MAb 1H9 was differentially expressed on well-differentiated and poorly differentiated human colon carcinoma cell lines suggesting that the expression, glycosylation and subcellular localization of this family of L-PHA-binding glycoproteins may correlate with the differentiation status of colon cancer cells and/or reflect biochemical changes characteristic of more progressive metastatic tumors.

Loss or truncation of β,1-6 branched oligosaccharides in metastatic tumor cells was found to have multiple effects on the cellular phenotype, including reduced cell invasion of BM (Yagel et al., 1989), enhanced TIMP gene expression (Korczak et al., 1993) and decreased cellular response to autocrine growth stimulation (VanderElst and Dennis, 1991). These observation suggest that simplification of the complex-type oligosaccharides expressed on tumor cells may affect the malignant or differentiated phenotype, in multiple ways including at the level of gene expression.

**Figure 1. The metastasis cascades**

The process of metastasis formation is a complex cascade of events, which include: (*A*) detachment and release of cells from the primary tumors and invasion of tumor cells into surrounding stroma, (*B*) transport of tumor cells into the lymphatics or the vasculature and survival in the circulation, (*C*) arrest at new location, (*D*) attachment and extravasation into the surrounding parenchyma and finally (*E*) establishment of a new growth at the new site. (Adapted from Nicolson, 1982; Tarin and Matsumura, 1994).

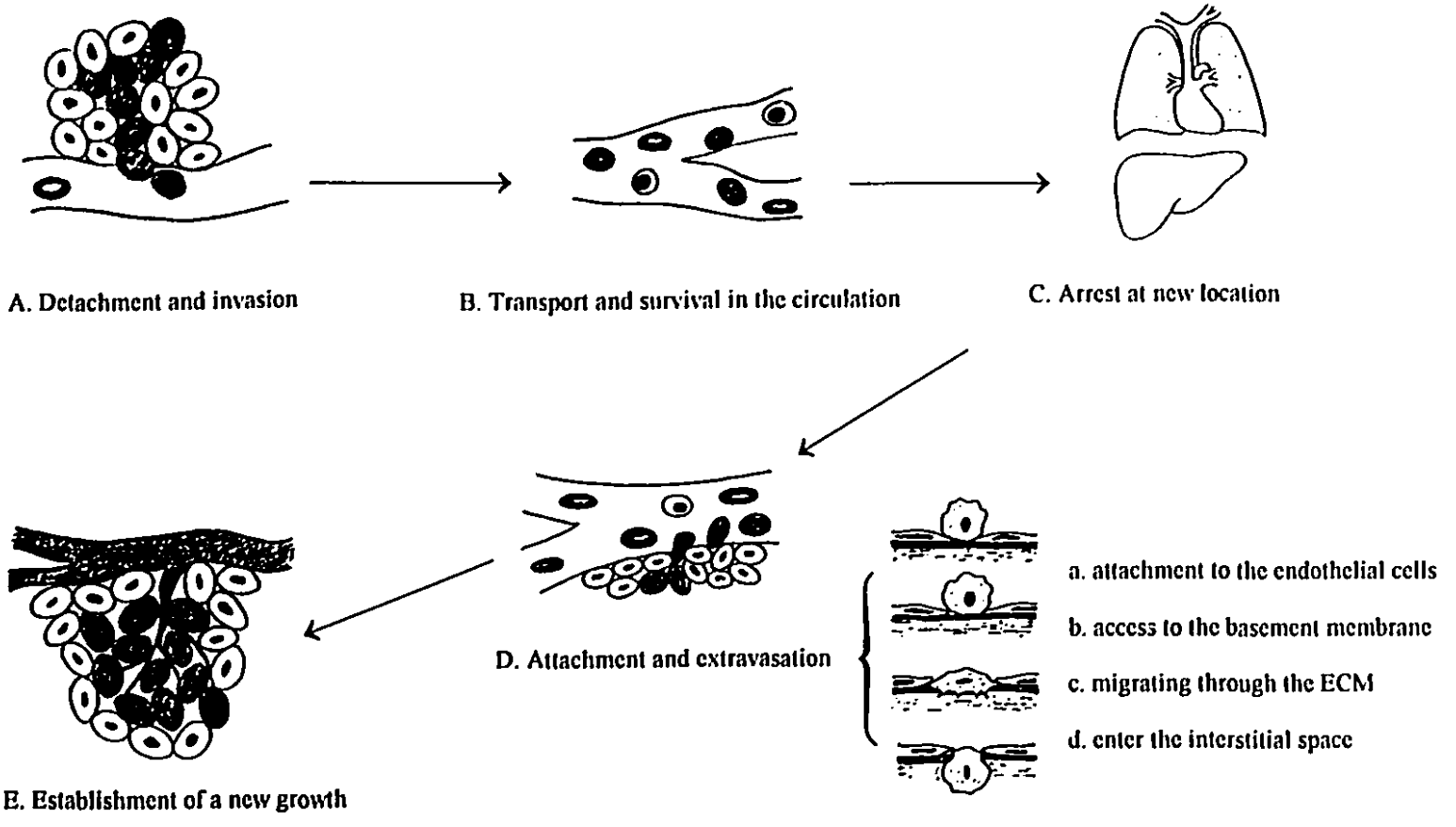



**Figure 1. The metastasis cascades**

## Table I: Integrins Expression and Cancer Metastasis (some examples)

| Integrins | Ligand | Expression | Adhesion/Metastasis | References |
|---|---|---|---|---|
| *positively-regulated:* | | | | |
| $\alpha_1\beta_1$ | collagen (Col) | melanoma | ↑ adhesion | Kramer et al., 1989 |
| $\alpha_2\beta_1$ | Col, laminin (LN) | Rhabdomyo-sarcoma (RDA2) | ↑ pulmonary metastasis | Chan et al., 1991 |
| $\alpha_3\beta_1$ | FN, Col, LN, epiligrin | human solid malignancies | ↑ metastases | Bartolazzi et al., 1994 |
| $\alpha_4\beta_1$ | VCAM-1 | melanoma | ↑ pulmonary metastasis | Okahara et al., 1994 |
| $\alpha_5\beta_1$ | FN (on cells) | TA3/St mammary carcinoma | ↑ hepatic metastasis | Kemperman et al., 1994 |
| | FN (on ECM) | melanoma | ↑ metastasis | Mortarini et al., 1992 |
| $\alpha_6\beta_1$ | LN | 1. colon carcinoma | ↑ hepatic metastasis | Lotz et al., 1990 |
| | | 2. melanoma | ↑ lung colonization | Ruiz et al., 1993 |
| $\alpha_6\beta_4$ | LN | TA3/Ha mammary carcinoma | ↑ liver metastasis | Kemperman et al., 1993 |
| $\alpha_{IIb}\beta_3$ | fibrinogen | B-16 melanoma | ↑ lung colonization | Chang et al., 1992 |
| $\alpha_L\beta_2$ | ICAM-1 | T-cell hybridoma | ↑ liver metastasis | Roossien et al.,1989,1994 |
| $\alpha_v\beta_1$ | VN, fibrinogen | melanoma | ↑ adhesion | Marshall et al., 1995 |
| $\alpha_v\beta_3$ | FN, VN | melanoma | ↑ lymphatic metastasis | Nip et al., 1992, 1995 |
| *negatively-regulated:* | | | | |
| $\alpha_2\beta_1$ | Col | colorectal adenocarcinoma | ↑ tumor differentiation | Pignatelli et al., 1990b |
| $\alpha_3\beta_1$ | FN, LN, Col (IV) | mouse skin carcinomas | ↓ tumor progression | Tennenbaum et al., 1992 |
| $\alpha_4\beta_1$ | VCAM-1 | B-16 melanoma | ↓ pulmonary metastasis | Qian et al., 1994 |
| $\alpha_5\beta_1$ | FN | transformed CHO cell | ↓ tumorigenicity | Giancotti et al., 1990 |

↑ : positively associated ↓ : negatively associated

# 2. Liver metastasis: Clinical, Anatomical and Biological aspects

## 2.1. Clinical significance of hepatic metastasis

The liver is one of the most common sites of metastasis in patients with malignant diseases. Almost all cancers from various primary sites metastasize to the liver. More common tumors include those of the gastrointestinal tract (colon, stomach, pancreas, etc.), melanoma, lung and breast carcinomas. Other tumors such as genitourinary cancers, sarcomas and lymphoreticular malignancies metastasize to the liver less often (Meyers and Jones, 1990). Liver metastases are often multiple and bilobar and cannot be easily diagnosed in their early stages. Once symptoms appear, the metastases are generally inoperable and respond poorly to chemotherapy resulting in poor prognosis. In general, patients with untreated hepatic metastases have median survival rates ranging from 6-18 months (Tranberg and Bengmark, 1994) and for patients with hepatic resection for metastases of colorectal carcinoma, the median survival rate is 24 months (Jatzko et al., 1995).

## 2.2. Prognosis and therapeutics of liver metastasis

The treatment of liver metastasis still remains very unsatisfactory since most patients do not have obvious physical findings or marked abnormal laboratory tests during the early stages of metastatic liver disease.

### 2.2.1. Surgical resection

In general, liver resection for metastatic tumor is indicated only when the cancer is of colorectal origin, the hepatic tumor is removable, and no extrahepatic disease is present

(Tranberg and Bengmark, 1994). The accumulated experience shows that the patients with colorectal cancer which have metastases limited to one lobe and the number of liver tumors is less than four can be successfully treated by surgical resection (Ballantyne and Quin, 1993). Unfortunately only about 5% of patients with colorectal cancer fall into this category and resection of hepatic metastases can therefore improve overall survival of patients with colorectal cancer by only 1-2%. Normally only 5-10% of patients with metastases only or predominantly in the liver will be amenable to potentially curative surgical resection. The 5-year survival rate of patients undergoing liver resection is approximately 30% and the 10-year survival rate is approximately 20% (Tranberg and Bengmark, 1994).

### 2.2.2. Chemotherapy

For the majority of patients with liver metastases, chemotherapy is the rational option since liver failure is often the result of widely disseminated disease. Chemotherapy is effective in some cancers (e.g. germ cell tumors and lymphoma), whereas in others the result is uncertain (Ravikumar and Steele, 1991).

#### 2.2.2. a. Systemic chemotherapy

Systemic chemotherapy has been used in patients with liver metastases derived from different advanced cancers. The major agent for therapy in patients with advanced colorectal cancer is 5-Fluorouracil (5-Fu). A combination of different chemical agents is generally more effective than a single agent. For patients with advanced colorectal cancers, an overall chemotherapy response rate of about 20% has been shown by both randomized and nonrandomized trials, but they do not prolong survival. In other

gastrointestinal cancers (except neuroendocrine tumors) and other tumors such as melanoma or lung cancer, the results are also disappointing. Breast cancer, gastrointestinal neuroendocrine tumors and germ cell tumors (testicular and ovarian) as well as lymphoreticular malignancies have shown some response to systemic chemotherapy alone or in combination with hormonal manipulation (Ravikumar and Steele, 1991).

**2.2.2. b.** Hepatic arterial infusion chemotherapy

The administration of chemotherapy by continuous hepatic arterial infusion (HAI) was introduced as a means of delivering cytotoxic drugs directly to the tumor by hepatic artery catheterization (Ramming and O'Toole, 1986). HAI is indicated when liver metastases are unresectable and extrahepatic disease is absent or minimal. The chemical agent of choice for regional infusion is usually 5-fluoro-2-deoxyuridine (FUDR), which has an 80-95% extraction rate during the first passage through the liver. This treatment was reported to cause a 20% regression of cancer and a 50% symptomatic improvement. A randomized trial demonstrated a high response rate compared with systemic treatment (Kemeny et al., 1987). However the true benefit of infusion therapy for survival still needs to be demonstrated by randomized trials (Tranberg and Bengmark, 1994).

**2.2.3.** Other forms of therapy

The external beam radiation may be used for palliative treatment, such as pain relief or liver size reduction, but survival rate is probably not affected (Sherman et al., 1978). Metastatic tumors may also shrink when using hepatic artery ligation or embolization, but the response is likely to be modest and temporary if it occurs at all (Blumgart and Allison, 1982). Liver transplantation has been used for metastatic

neuroendocrine tumors in combination with additional resection of the pancreatic primary tumor, but only 6% of patients had a 2-year survival (Alsina et al., 1990).

### 2.2.4. Experimental therapy

The application of cryotherapy has been tried with the intent to cure surface lesions, and it can be an effective alternative to liver resection in patients with marginal hepatic function (Ravikumar et al., 1987). Biologic response modifiers (interleukin-2 and lymphokine-activated killer cells) used as immunotherapy for the treatment of different tumors at various anatomic sites may be effective against metastases of certain histologic types of tumors such as renal cell cancer and melanoma and have been used as adjuvants to surgical resection (Schwarz et al., 1989).

To summarize, despite some progress in the treatment of liver metastases, elongation of patients life has not yet been achieved. The design of more effective therapeutic reagents for hepatic metastasis is urgently needed. This can only be achieved through a better understanding of the biology of the process of liver colonization.

## 2.3. Anatomical aspects of liver metastases

### 2.3.1. Blood supply of the liver

The normal human liver has a double blood supply: the portal vein and the hepatic artery. The portal vein is formed by the junction of the superior mesenteric and splenic veins, which provides about three fourths of the liver's blood supply (Meyers and Jones, 1990) (Fig. 2A, page 47). The portal trunk divides into left and right hepatic branches in the portal fissure and these further divide into small veins and venules which finally enter

the hepatic sinusoids (Fig. 2B, page 47). The hepatic artery coming from the celiac axis, supplies one fourth of the liver with arterial blood. It also enters the liver through a fissure and then forms a plexus and empties into the sinusoids. The sinusoids enter the central venules or terminal hepatic venules (THV) (Fig. 4, page 49) before draining into hepatic vein and finally to the inferior vena cava (Fig. 2A, page 47) (Barberá-Guillem and Vidal-Vanaclocha, 1991).

**2.3.2.** Hepatocytes

Hepatocytes comprise about 60% of the cells in the liver. They are polygonal and approximately 30 μm in diameter. Hepatocytes form single layers (cell plates) between minute vascular channels called the sinusoids (Fig. 2B, page 47 and Fig. 4, page 49). The hepatocyte has three surfaces: one facing the sinusoid and space of Disse (see below), the second facing the canaliculus of the bile ducts and the third facing neighboring hepatocytes (Fig. 3, page 48). There is no BM around the hepatocytes (Sherlock and Dooley, 1993).

**2.3.3.** Hepatic sinusoids and cells

The micro-vascular channels between single layers of hepatic parenchymal cells are called sinusoid (Fig. 2B, page 47). The flat fenestrated endothelial cells line and form the walls of the sinusoid (Fig. 3, page 48). The sinusoidal cells which include endothelial cells, Kupffer cells, fat storing cells (Ito cells) and pit cells as well as various subpopulation of lymphoid cells form this functional and histological unit together with the sinusoidal aspect of the hepatocytes (Sherlock and Dooley, 1993). Kupffer cells overlap the endothelial cells loosely and attach to the sinusoidal wall. The hepatocyte membrane microvilli project through this fenestra for ready exposure to sinusoidal contents. The endothelial fenestra

have no diaphragm and are freely permeable to low and high molecular weight substances in the sinusoidal flow. This also allows easy access for tumor cells invading the hepatic parenchyma.

**2.3.3. a.** Kupffer cells

Kupffer cells are hepatic resident macrophages which are involved in the defense system of the organ under various physiological and pathological conditions (Philips, 1989). They are derived from blood monocytes and have only a limited capability for cell division, but are highly mobile and can attach to the endothelium. Kupffer cells have specific membrane receptors for ligands including the Fc portion of immunoglobulin and the C3b component of complement, which are important for antibody dependent cellular cytotoxicity and complement mediated cytotoxicity. They can endocytose old cells, foreign particles, tumor cells, bacteria, yeasts, viruses and parasites providing a natural defence mechanism.

An example of Kupffer cells involved in tumoricidal activity was shown by Heuff and his colleagues. Using a liver metastatic rat colon carcinoma cell line, they demonstrated that various populations of macrophages and resident Kupffer cells may be involved in the host response against tumor cell deposits in the liver (Heuff et al., 1993). Kupffer cells were also found to be activated by co-culture with tumor cells and were subsequently able to lyse and phagocytose the cancerous cells (McCuskey et al., 1994). These Kupffer cells could be observed in contact with tumor cells not only in sinusoids but also in tumor forming foci within the hepatic parenchyma although the metastases were still observed since tumor cells can escape from Kupffer cells. This evidence indicated that

Kupffer cells interact with tumor cells and partially endocytose them during metastasis formation.

**2.3.3. b.** Sinusoidal endothelial cells

Endothelial cells line the sinusoid to form the fenestrated wall surrounding the sinusoidal lumen (Fig. 3, page 48). This fenestrae regulate the exchange of fluids and particulate matter to and from the space of Disse and hepatocytes (Wisse et al., 1985). Sinusoidal endothelial cells are the first barrier encountered by tumor cells entering the sinusoids from the blood. Two distinct types of endothelial cells differing in their structure and specific lectin binding patterns have been identified in different acinar zones. Zone 1 endothelial cells have a high concentration of certain lectin-binding carbohydrate residues (e.g. N-acetyl-neuraminic acid, galactose, N-acetyl-galactosamine, mannose and glucose) and more binding sites for the lectin wheat germ agglutinin WGA which has an affinity for N-acetyl-glucosaminyl residues and N-acetyl-glucosamine oligomers as compared with the endothelial cells in Zone 3 (Barberá-Guillem et al., 1989; 1991) (see 2.3.6). In most species, endothelial cells of the liver sinusoid have no continues BM (Barberá-Guillem and Vidal-Vanaclocha, 1991).

**2.3.3. c.** Pit cells

Pit cells are highly mobile, located in the lumen of the hepatic sinusoids attached to the liver endothelial or Kupffer cells by cytoplasmic processes. They show characteristic granules and rod-cored vesicles. They have natural killer activity to certain tumor cells and virally infected hepatocytes and have many features in common with large granular

lymphocytes. They therefore may act in the liver as a first line of defense against neoplasia, metastasis, and viral infections (Geerts et al., 1990).

**2.3.3. d.** Fat storing (Ito) cells

The fat storing cells (Ito cells) lie in the space of Disse in close contact with hepatocytes on one side, and endothelial cells on the other, but are also distributed in the hepatic parenchyma between hepatocytes (Fig. 3, page 48). They store excess vitamin A and other fat soluble vitamins. Ito cells can migrate to zone 3 where they change into myofibroblasts in the presence of damaged hepatocytes and may be involved in repair mechanisms. In culture, Ito cell can secrete a variety of matrix components, such as types I, III and IV collagen, FN and laminin (Ramadori, 1991). These proteins may be involved in the promotion of tumor growth *in vivo*.

**2.3.4.** The space of Disse

The space between the thin endothelial lining of the sinusoidal lumen and hepatocytes is the perisinusoidal space of Disse (Fig. 3, page 48). Within the space of Disse, Ito cells, fibroblasts and neurons, ECM proteins and electrolytes can be found as well as small amounts of tissue fluid which flows outwards into lymphatic channels in the portal zones (Sherlock and Dooley, 1993). The ECM in the space of Disse has several unique features: type I collagen which is in direct contact with hepatocytes and endothelial cells forms the scaffold of the hepatic lobule while type IV collagen is not associated with laminin and does not form part of a BM (Martinez-Hernandez, 1984).

### 2.3.5. Extracellular matrix proteins

Extracellular matrix molecules including FN, type I and type IV collagen, laminin, heparin sulfate, entactin and some cell associated proteoglycans can be found in or around the space of Disse. Basement membrane structures are present around the portal and central veins and surround the bile duct epithelium (Abrahamson and Caulfield, 1985). There is no continuous BM underlying the sinusoidal endothelial cells. The hepatocytes and endothelial cells are separated by an ECM containing mainly FN, some type I collagen and type IV collagen. Fibronectin is the most prominent component of the ECM in the space of Disse (Martinez-Hernandez, 1984).

### 2.3.6. Hepatic acinar units and their zones

The liver acinus is a small parenchymal mass arranged around the portal axis which consists of a terminal portal venule, terminal hepatic arteriole, bile ductule, lymph vessels and nerves (Fig. 4, page 49). It lies between two or more terminal hepatic venules (THV) also called central veins. Hepatic blood flows from the terminal portal venules (TPV) into the sinusoids and comes into contact with hepatocytes within the unit until it drains into the THV. Three microcirculatory zones have been identified according to the blood supply of first, second and third quality with regard to oxygen and nutrient contents. Zone 1 represents the area close to the supplying portal axis vessels, zone 3 corresponding to the area that is most distant from the supplying vessels but close to the THV and zone 2 is the border area between zone 1 and zone 3 (Fig. 4, page 49) (Sherlock and Dooley, 1993).

There are some notable differences between zones 1 and 3. In zone 1, the sinusoids are more winding and anastomotic, have a higher concentration of Kupffer cells and their

endothelial cells are less porous and more adhesive because they express more carbohydrate molecules (see 2.3.3. b) (Barberá-Guillem et al., 1991). These differences have been utilized to distinguish between the two zones and follow tumor cell migration through the liver following entry through the portal vein.

## 2.4. Microenvironment of liver and liver metastasis

As is the case for other organs, the potential of cancer cells to metastasize to the liver is dependent on multiple specific interactions between the tumor cells and different constituents of the liver microenvironment including the microvascular endothelium, extracellular matrix proteins, hepatocytes and paracrine growth factors.

### 2.4.1. Cancer cell arrest in the liver.

After detachment from the primary malignant lesion following passage through the circulation and arrival in the liver, the surviving tumor cells are retained along the entire sinusoidal pathway and are arrested mostly in acinar zone 1 (Barberá-Guillem and Vidal-Vanaclocha, 1991). This may be due to the different features of these two specific area as mentioned above, namely the higher degree of anastomosis, the higher number of Kupffer cells which may act as sinusoidal sphincters and the higher concentration of carbohydrate residues on the sinusoidal endothelial cells which lead to more adhesiveness in zone 1. Tumor cell interaction with the sinusoidal endothelium in particular, appears to play a role in tumor cell arrest. This has been observed microscopically and also supported by functional *in vitro* assays (Tressler et al., 1993; 1994; Brodt et al., 1994).

2.4.2. Tumor - endothelial cell interaction

Tumor cell adhesion to the endothelial cells is the early step in the extravasation of metastatic tumor cells from the blood stream into the tissue space of the liver. This interaction is thought to be similar to that occurring between lymphocytes and cytokine - activated vascular endothelial cells during inflammation. Lymphocytes often infiltrate the liver during hepatic inflammation. ICAM-1 was found to be expressed on the sinusoidal endothelium or on portal and hepatic endothelium in human liver (Adams et al., 1989) which is recognized by LFA-1 expressed on lymphocyte and can therefore mediate lymphocyte attachment to endothelial cells during inflammation.

Several investigations have identified specific adhesion receptors on endothelial cells which may mediate tumor cell adhesion to the liver endothelium. The most discussed was E-selectin which can be expressed on endothelial cells and can interacted with its ligands on the tumor cell surface.

Using human umbilical vein endothelial cells (HUVEC) it was shown that E-selectin which can be induced on endothelial cells by cytokines such as TNF-$\alpha$ and IL-1 can mediate adhesion to the carbohydrate ligand $SLe^X$ which is expressed on human colon carcinoma cells (HCC) (Lauri et al., 1991). This adhesion may contribute to liver metastasis since HCC are metastatic mainly to the liver. It was also reported that the HCC cells KM12-HX which express high levels of $SLe^X$ colonized to the liver more efficiently than KM12-LX cells which are low expressors when the cells were injected intrasplenically into nude mice. Under *in vitro* condition, KM12-HX cells demonstrated a greater degree of adhesion to cytokine - activated HUVEC than KM12-LX cells. The

adhesion was partially inhibited by antibodies specific for E-selectin. Moreover, treatment of KM12-HX cells with an inhibitor of O-linked carbohydrate chain elongation reduced the adhesion of KM12-HX cells to HUVEC indicating that O-linked carbohydrate chains on the HCC cell surface played a significant role in the adhesion (Izumi et al., 1995). An other group showed that the adhesion of HCC cells to HUVEC was significantly inhibited by pretreatment with anti-E-selectin antibody as well as antibody to the sialylated carbohydrate antigen SPan-1. The adhesion was also inhibited by pretreatment with neuraminidase (Yamada et al., 1995). These results suggested that the SPan-1 antigen plays a significant role in adhesion of HCC cell to endothelial cell, and sialylation of the terminal structure of the carbohydrate antigen was important in the adhesion of HCC to endothelial cells.

Using the H-59 tumor model it was shown that hepatic sinusoidal endothelial cells express E-selectin in response to TNFα. Furthermore, anti-E-selectin antibodies significantly inhibited H-59 cell adhesion to hepatic sinusoidal endothelial cells in the presence of TNFα. *In vivo* studies conformed the relevance of E-selectin to liver metastasis by showing that anti-E-selectin antibodies blocked experimental liver metastases. In this study, a mucin-associated carbohydrate structure with fucose and sialic acid in terminal positions was involved in the adhesion (Brodt et al., 1994, manuscript submitted).

Recently, Tressler and associates reported that annexin II and VI are expressed on the surface of liver metastatic murine RAW 117 large cell lymphoma cells and can mediate part of the $Ca^{++}$-dependent RAW 117 cell adhesion to liver microvessel endothelial cells

(Tressler et al., 1993, 1994) indicating these molecules may also be involved in liver colonization by murine lymphoma cells.

**2.4.3.** Tumor cell interaction with the ECM

Tumor cell invasion through different ECM barriers is another major step in the metastatic process. Since the hepatic sinusoidal endothelium lacks a continuous BM (see 2.3.5), tumor cells in the sinusoid can invade directly into the subendothelial space. During their invasion, tumor cells can either interact with ECM components e.g. laminin or FN (Rusciano et al., 1991) through tumor cell surface adhesion molecules and/or express proteolytic enzymes to degrade ECM and therefore enhance their invasion and migration.

Fibronectin is the most prominent component of the ECM in the space of Disse. Tumor cell adhesion to FN might lead to tumor cells invasion into the liver parenchyma (Nicolson, 1988b). It has been reported that a fusion fragment of the heparin-binding domain of human FN significantly inhibited lymphoma cell adhesion to substrates precoated with FN, laminin and reconstituted BM (Matsumoto et al., 1991). Heparin and MAb against the heparin binding domain of FN inhibited tumor cell interaction with the fusion polypeptide-substrate *in vitro*, and also blocked liver metastasis when co-injected with the tumor cells. This may have been due partly to interference with the adhesion of tumor cells to ECM or BM components by this heparin-binding domain-dependent mechanism. The positive correlation between perisinusoidal laminin expression and liver metastasis was demonstrated by Wewer and Albrechtsen (Wewer and Albrechtsen, 1992). They found that liver metastatic tissue showed high staining for laminin. Moreover, viable

tumor cells bind specifically to frozen sections of liver tissue containing perisinusoidal laminin. This suggested that the perisinusoidal laminin may be involved in tumor cell metastasis to the liver. Recently, the overexpression of stromelysin-3, a newly identified member of the matrix-degrading MMP family on human colorectal tumor has been reported. This overexpression of stromelysin-3 is correlated with the progression of human colorectal tumors toward local invasion and may be involved in liver metastasis (Porte et al., 1995).

Liver metastatic H-59 cells have been reported to express high levels of gelatinase A and the cysteine proteinase cathepsin L as well as lower levels of cathepsin B (Brodt et al., 1992). These enzymes are known to be involved in tumor cell invasion. Recently it was found that an inhibitor of cysteine proteinase could block experimental liver metastasis (Navab et al., 1995) suggesting that these enzymes may degrade liver ECM and thereby promote liver colonization by H-59 cells.

### 2.4.4. Tumor cell interaction with the hepatocytes

Tumor cell adhesion to hepatocytes has been observed in several experimental models of liver metastasis. For example, hepatocytes isolated from normal rat liver have been found to express ICAM-1. This molecule could bind LFA-1 which is expressed on liver metastatic T-cell hybridoma cells. A MAb directed to ICAM-1 could inhibit the adhesion of T-cell hybridoma cells to the hepatocytes. It was also found that tumor cell invasion was associated with substantial redistribution of LFA-1 on the T-cell hybridoma

cell surface and also with ICAM-1 redistribution on the hepatocyte surface (Meijne et al., 1994).

Integrin $\alpha_6\beta_4$ has been identified as a receptor on TA3/Ha mammary carcinoma cells which is involved in the adhesion of these cells to the hepatocytes. Anti- $\alpha_6\beta_4$ Fab fragments generated from a polyclonal serum against TA3/Ha cells inhibited TA3/Ha - hepatocyte adhesion (Kemperman et al., 1993) suggesting that $\alpha_6\beta_4$ mediated adhesion to hepatocytes may contribute to liver colonization. Recently it has been reported by the same group that cells of another subline of mammary carcinoma TA3/St which express integrin $\alpha_5\beta_1$ utilize this receptor to interact with hepatocyte surface associated FN. This interaction may be necessary for liver metastases formation (Kemperman et al., 1994).

In addition, the isolated tumor cell surface oligosaccharide T (Thomsen-Friedenreich) antigen and its immediate precursor Tn antigen have also been implicated in adhesion to rat hepatocytes and Kupffer cells (Schlepper-Schafer and Springer, 1989). The interaction of these specific pancarcinoma epitopes T and Tn on cancer cells with Gal/GalNAc receptors on rat hepatocytes and Kupffer cells may play a role in liver metastasis of some carcinomas.

The ultrastructure of the interaction between tumor cells and hepatocytes has been studied *in vitro* using a rat hepatoma subline which can invade a primary culture of adult rat hepatocytes. It was found that the tumor cells adhered to the hepatocytes with several types of junctional structures. An early event in the invasive process was an apparent cytoplasmic fusion between the tumor cells and the hepatocytes. It was suggested that this

event may be of functional significance in hepatic invasion *in vivo* (Igarashi and Kawaguchi, 1993).

**2.4.5.** Paracrine growth regulation and liver metastasis

The ability of tumor cells to grow in the liver is also regulated by organ-derived growth factors. These paracrine growth factors can be produced by the hepatocytes, sinusoidal endothelial cells, Kupffer cells or fibroblasts. For example, a liver extract was found to promote the growth of liver metastasizing embryonal carcinoma cells (Rusciano et al., 1991). The liver - metastasizing melanoma subline B16-L8 was also found to respond in a specific manner to growth stimulation mediated by hepatocytes while the lung colonizing subline B16-F10 did not have this response (Sargent et al., 1988). In addition, the liver - colonizing subline of the RAW117 large -cell lymphoma was reported to be growth stimulated by the complement component C3b present in medium conditioned by mouse hepatic sinusoidal endothelial cells (Hamada et al., 1993).

Transforming growth factor alpha (TGF-α) is produced by hepatocytes in response to trauma, as a physiological regulator of liver regeneration (Herlyn et al., 1990). The TGF-α is a ligand of the epidermal growth factor receptor (EGF-R) which is expressed on liver metastatic human colon carcinoma cells and it was suggested that it may be responsible for TGF-α mediated stimulation of the carcinoma cells (Radinsky, 1993).

Hepatocyte growth factor (HGF), also called scatter factor, is synthesized and secreted by different cell types, but also produced by liver endothelial, Kupffer, fibroblast and Ito cells. It binds to its receptor the c-met proto-oncogene expressed on hepatocytes

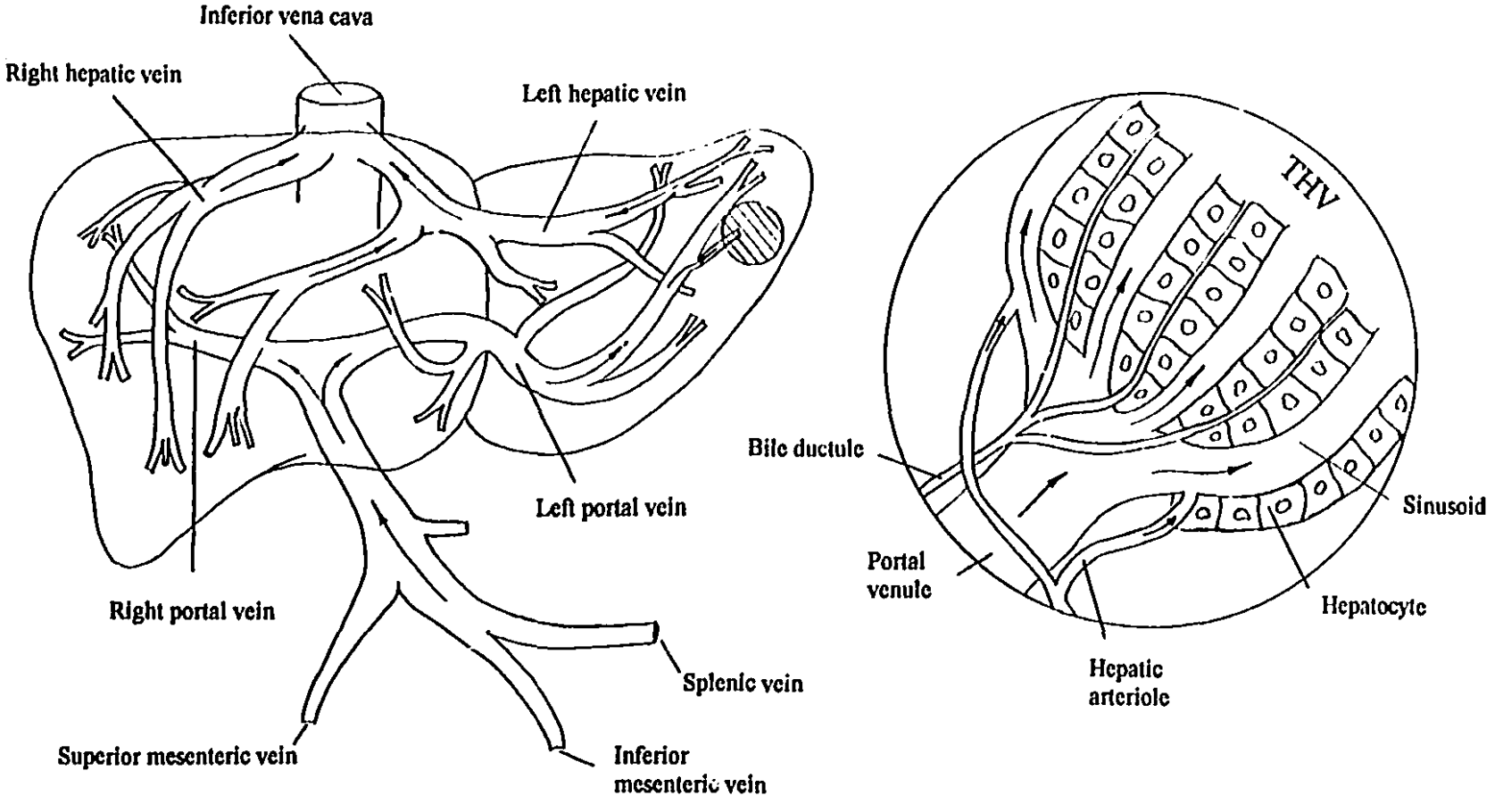
and other cells and can induce cell motility and regulate growth (Jiang et al., 1993a). The HGF receptor c-met protein is also highly expressed on malignant cells, such as colon cancer cells (Jiang et al., 1993b). The stimulation of colorectal carcinoma cells by liver-derived HGF may play a role in the metastasis of this malignancy to the liver.

Insulin-like growth factor-1 (IGF-1) has been found in medium conditioned by mouse hepatocytes. This hepatocyte-derived IGF-1 was found to mediate H-59 cell proliferation through the IGF-1 receptor expressed on H-59 cells, and a monoclonal antibody to IGF-1 could block the IGF-1- induced mitogenic activity *in vitro* (Long et al., 1994). Furthermore H-59 cells expressing an antisense mRNA to the IGF-1 receptor failed to give rise to liver metastases *in vivo* indicating that IGF-1 was involved in tumor H-59 metastasis to the liver (Long et al., 1995).

In summary, liver metastasis is a complex process involving multiple host tumor interactions such as the interaction of tumor cells with hepatic endothelial cells, with ECM proteins and with hepatocytes. Many of the molecules mediating these interactions remain to be positively identified.

**Figure 2. The hepatic circulation**

A. The hepatic and portal veins. The portal vein is formed by the junction of the superior mesenteric and splenic veins (merged by inferior mesenteric vein). It divides into left and right hepatic branches in the portal fissure and these further divide into small veins and venules which finally enter the hepatic sinusoids. The sinusoids enter the central venules or terminal hepatic venules before draining into hepatic vein and finally to the inferior vena cava. B. Microvascular bed of the liver (inset). The hepatic arteries and hepatic portal venules empty into the hepatic sinusoids. Arrows indicate the direction of the blood flow. (Adapted from Meyers and Jones, 1990; Mathie et al., 1994).




A. The hepatic and portal veins

B. Microvascular bed of the liver

**Figure 2. The hepatic circulation**

**Figure 3. Topography of the hepatocytes, sinusoids and the perisinusoidal space of Disse**

Hepatocytes form single layers (cell plates) between minute vascular channels called the sinusoids. Each hepatocyte has three surfaces: one facing the sinusoid and space of Disse, the second facing the canaliculus of the bile ducts and the third facing neighboring hepatocytes. The sinusoids form the micro-vascular channels between single layers of hepatic parenchymal cells. The flat fenestrated endothelial cells line and form the walls of the sinusoid. The hepatocyte membrane microvilli project through this fenestra for ready exposure to sinusoidal contents. The sinusoidal cells include the endothelial cells, Kupffer cells, fat storing cells (Ito cells) and pit cells as well as various subpopulation of lymphoid cells. The space between the thin endothelial lining of the sinusoidal lumen and hepatocytes is the perisinusoidal space of Disse.

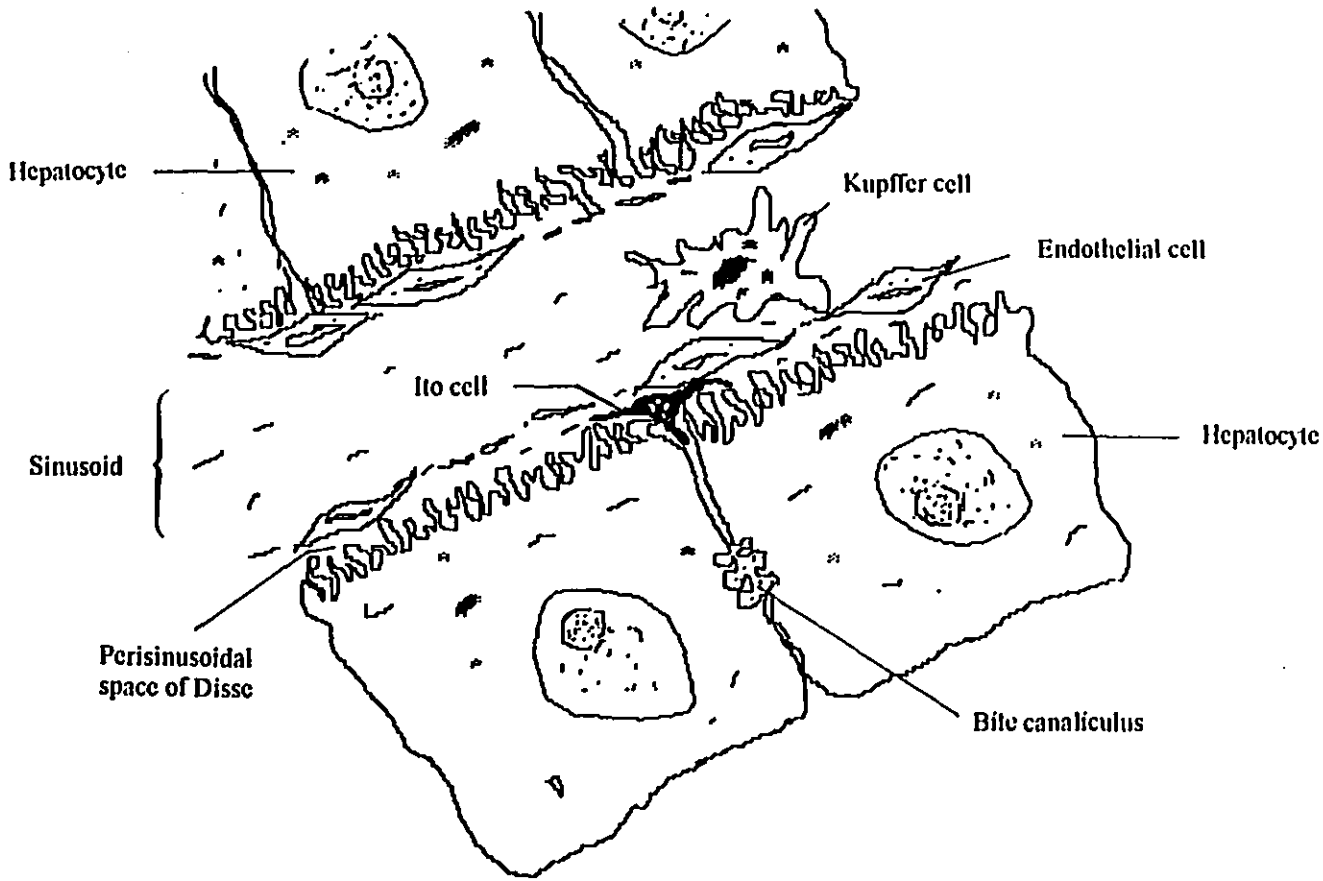



**Figure 3. Topography of the hepatocytes, sinusoids and the perisinusoidal space of Disse**

**Figure 4. The liver acinar unit and the three microcirculatory zones**

The liver acinus is a small parenchymal mass arranged around the portal axis which consists of a terminal portal venule, terminal hepatic arteriole, bile ductule, lymph vessels and nerves. It lies between two or more terminal hepatic venules (THV) also called central veins. Hepatic blood flows from the terminal portal venules (TPV) into the sinusoids and comes into contact with hepatocytes within the unit until it drains into the THV. Three microcirculatory zones have been identified according to the blood supply of first, second and third quality with regard to oxygen and contents of nutrients. Zone 1 represents the area close to the supplying portal axis vessels, zone 3 corresponding to :he area that is most distant from the supplying vessels but close to the THV and zone 2 is the border area between zone 1 and zone 3. Arrows indicate the direction of the blood flow. (Adapted from Meyers and Jones, 1990).

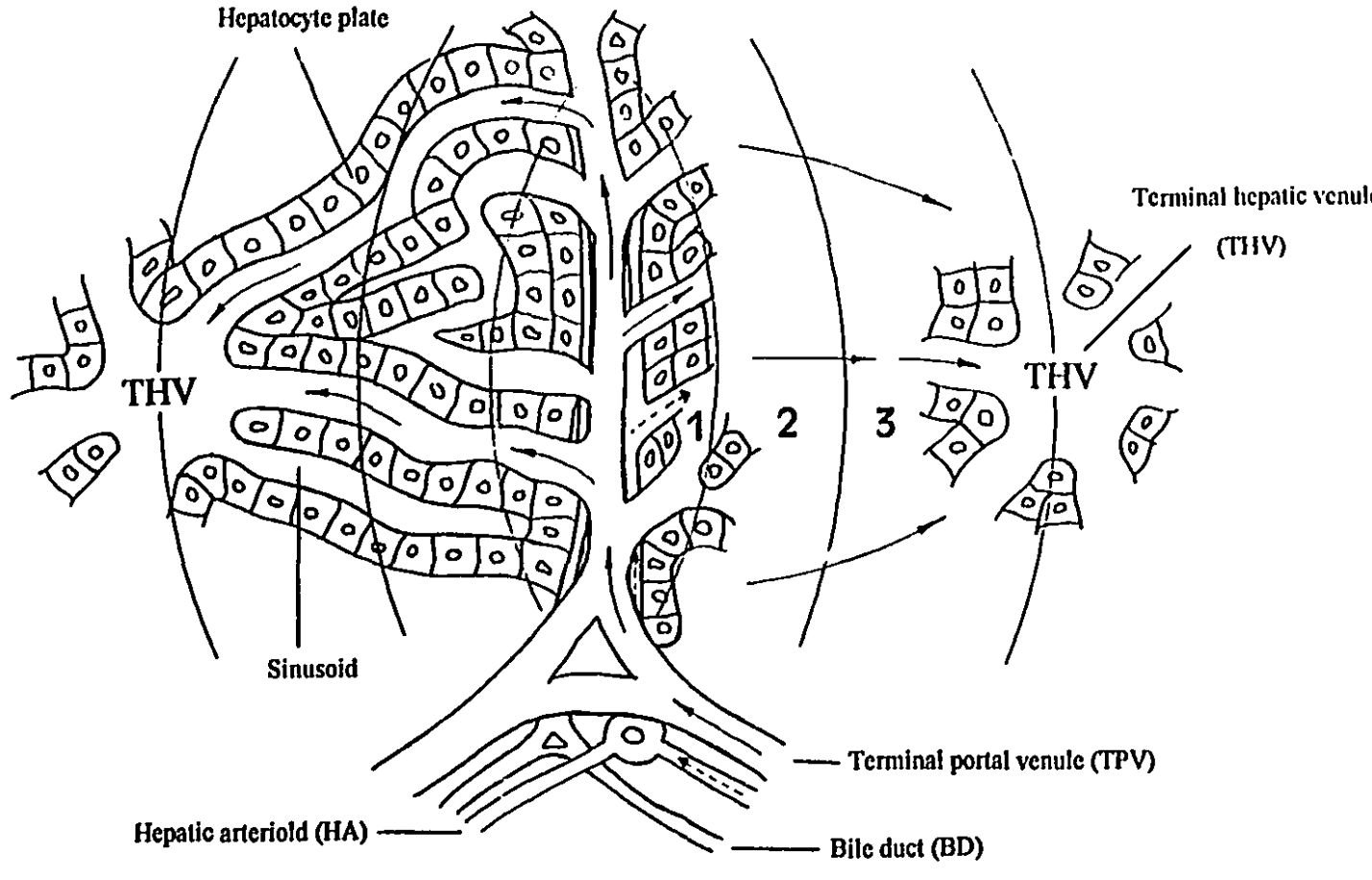



**Figure 4. The liver acinar unit and the three microcirculatory zones**

# 3. Monoclonal antibodies and cancer therapy

## 3.1. General Introduction

### 3.1.1. Significance of antibodies in cancer therapy

Antibodies, the serum immunoglobulins (Ig), are components of the humoral immune system and are synthesized by B-lymphocytes in response to antigens as part of an active immune defense mechanisms (Roitt, 1991). With the development of monoclonal antibodies by Köhler and Milstein in 1975, providing a method for unlimited production of high affinity, highly specific antibodies (Köhler and Milstein, 1975), the potential utilization of antibodies for diagnosis and treatment of neoplastic disease has become both feasible and attractive (Schwartz et al., 1992). Antibodies can have several possible therapeutic effects on the cancer cells. They can be used to activate the host immune system to destroy the tumor cells, can regulate growth and differentiation of tumor cells and can block adhesion of tumor cells to host cells and the extracellular matrix. They can also be used as delivery molecules, targeting potentially cytotoxic agents to tumor sites (Dillman, 1991).

### 3.1.2. HAMA and humanized MAb

Although murine MAbs have been used in cancer therapy, the major obstacle to long-term administration of murine MAbs to humans is the development of immune responses by the host especially if multiple administrations over an extended period of time are required to achieve a therapeutic effect

(LoBuglio et al., 1989). This response is mainly due to the host immune system reacting against determinants present in the Fc portion of murine Ig (Reynolds et al., 1989). The human anti-mouse immunoglobulin response known as "Human Anti-Mouse Antibody" or HAMA, can potentially influence the biological activity of the antibodies, diminishing the clinical response and can also induce adverse allergic reactions (Fagnani, 1994).

However, some approaches have been used to reduce this host immune response. These include the production of potentially less immunogenic MAb fragments and the development through genetic engineering of "humanized " MAb (Morrison and Oi, 1984). This technique is based on development of mouse/human chimeric MAb by joining the Ig variable domain from mouse MAb to the human Ig constant domain (Winter and Milstein, 1991). This chimeric "humanized" MAb is less immunogenic in cancer patients than murine MAb. Some of the chimeric MAbs have been administered to cancer patients, and a marked reduction in the HAMA response and a longer half-life of MAb have been observed (Hale et al., 1988; LoBuglio et al., 1989).

### 3.1.3. Functions of antibody fragments

Antibodies can play an indirect role in the host immune response by participation in antibody dependent cell mediated cytotoxicity (ADCC) and complement mediated cytotoxicity (CMC), mechanisms which depend on the Fc domain of the immunoglobulins, or they may inhibit tumor cell growth directly by

interacting with molecules such as growth factor or adhesion receptors through the Fab domains, blocking their functions (Houghton and Scheinberg, 1991). Genetic engineering techniques have been used to produce cDNA fragments coding for single chain antigen - binding proteins (Fv) consisting of the variable regions of the light and heavy chain genes which are linked and expressed in bacterial or eukaryotic vectors (Skerra and Pluncкthun, 1988; Reichman et al., 1988).

The advantages of the Fab fragment and the engineered antibodies in cancer therapy include: increased tumor penetration, rapid plasma clearance with a possibly decreased toxicity due to radionucleotides or immunotoxins conjugated to the antibodies, and decreased immunogenicity due to a lack of the Fc domain (Schwartz et al., 1992).

### 3.2. Strategies for therapeutic application of MAb

A number of distinct approaches for cancer therapy with MAb have been developed. Each approach requires certain prerequisite information about the individual antibody-antigen system such as antibody specificity, antibody class and isotype, antibody avidity or the structure and function of the antigen. This information is normally derived through a series of preclinical studies which are designed to address the following questions: (a). the ability of MAb to mediate killing of target tumor cells in the presence of immune effector cells (ADCC) or in the presence of complement (CMC) (Goodman et al., 1990). (b). the direct biological effects of MAb on target cells, such as their effect on tumor cell growth

and proliferation (Aboud-Pirak et al., 1988). (c). cytotoxic/cytostatic effects of the MAb when conjugated to anti-cancer agents (Goldenberg, 1989), and (d). the direct effect of MAb on target cell adhesion to host cells or organs (Zhu et al., 1991, 1992). These *in vitro* assays can be complemented by *in vivo* studies. These preclinical studies can determine the strategies to be used for human trials. The types of antibodies and the approaches which have been utilized to date can be summarized as follows:

**3.2.1.** Destruction of tumor cells by immune mechanism

**3.2.1. a.** Antibody Dependent Cell - mediated Cytotoxicity (ADCC)

Host immune defense cells such as neutrophils, macrophages and natural killer cells can bind antibody coated tumor cells by recognizing the Fc portion of the Ig molecule through their Fc receptors (Wawrzynczak and Thorpe, 1991). This leads to tumor cell killing through phagocytosis (by macrophages) or direct cytolysis (by NK cells). This was demonstrated by Herlyn et al who observed tumor cell lysis when anti - tumor MAbs were incubated with effector cells such as splenocytes from nude mice (Herlyn et al., 1979) and supported by subsequent *in vivo* experiments (Herlyn and Koprowski, 1982). Numerous other investigators reported similar effects both *in vitro* and *in vivo* (see below). The IgG2a and IgG3 subclasses of mouse MAb have been reported to be most effective in mediating ADCC mechanisms (Hellström et al., 1986; Woodhouse and Morgan, 1989).

**3.2.1. b.** Complement Mediated Cytotoxicity (CMC)

Some murine MAbs can activate complement by binding the C1q component of complement and thereby initiating a complement activating cascade. The "membrane attack complex" formed by the terminal complement components inserts into the cell membrane inducing cell lysis and destruction. Studies using mouse serum as a source of complement demonstrated that IgM is the most common activator of complement, while IgG2a, IgG2b and IgG3 were of medium or low potency. Activation of complement mediated cytolysis by IgG1 antibodies was only occasionally observed (Neuberger and Rajewski, 1981).

**3.2.1. c.** ADCC and CMC in experimental and clinical trials

The induction of ADCC and CMC by cell bound MAb has been reported by different investigators using diverse tumor models. The following are some examples.

Several of the early studies have been reported by Hellström and his associates. This group produced three mouse MAbs (IgG3) which recognized a $G_{D3}$ ganglioside antigen expressed on most human melanoma cells. These antibodies could mediate ADCC when combined with normal human lymphocytes. One of these antibodies also killed melanoma cells in the presence of human serum as a source of complement while another was found to inhibit human melanoma growth in nude mice (Hellström et al., 1985). It was postulated that this inhibition may have been due to antibody induced ADCC.

One of the antibodies MAb L6 (IgG2a) also recognized a ganglioside antigen that is expressed on most human carcinomas such as lung, breast, colon and ovary (Hellström et al., 1986). This MAb could lyse L6 antigen-positive human tumor cells in the presence of human lymphocytes or human serum *in vitro* and inhibit the outgrowth of a L6 antigen-positive human tumors in nude mice. This anti-tumor effect was tested on patients with breast, colon, ovarian and lung cancer. One patient with recurrent breast cancer achieved a pathologically confirmed complete remission after treatment with MAb L6 (Goodman et al., 1990). This anti-tumor effect suggested that unmodified L6 could be used in breast cancer therapy.

### 3.2.2. Regulation of tumor growth by MAb

#### 3.2.2. a. MAb to growth factor receptors

Animal experiments have shown that treatment of tumor - bearing mice with MAbs directed against growth factor receptors expressed on the tumor cell surface could significantly reduced tumor cell growth. Epidermal growth factor (EGF) or transforming growth factor α (TGF-α) are important regulators of the survival and growth of squamous cell lung carcinomas (Wendler and Ozanne, 1984) and ductal breast cancer cells (Bates et al., 1988). These growth factors share a common receptor - the EGF receptor. Sela and his group reported that anti-EGF receptor antibodies reduced human epidermoid carcinoma cell colony formation *in vitro*. When this effect was tested *in vivo* in nude mice, the anti-tumor

activities observed included retardation of tumor growth, a reduction in the number of experimental lung metastases and the prolongation of survival time. The F(ab)2 fragment of this anti-EGF receptor antibodies had the same effects suggesting that the anti-tumor effects were not due to immune mechanism such as ADCC and CMC (Aboud-Pirak et al., 1988). In a dose response study, an anti-EGF receptor MAb labeled with $^{111}$In used in a phase I trial in patients with inoperable squamous cell lung cancer showed no toxicity to patients and MAb uptake by the tumor was observed in a specific and dose-dependent fashion (Divgi et al., 1991). It was concluded that treatment with a MAb against EGF receptor is safe at the doses and schedules used.

The transmembrane tyrosine kinase protein - related molecule p185 (HER-2/neu oncogene product) was reported to be overexpressed in tumors derived from breast and ovarian cancer patients with poor prognosis (Berchuck et al., 1990). It has been reported that anti-p185 MAbs of the IgG1, IgG2a and IgG2b isotypes could inhibit the tumorigenicity of neu-transformed cells. Depletion of complement and macrophages in the tumor bearing animals did not affect this inhibition suggesting that ADCC and CMC mechanisms were not involved (Drebin et al., 1988). These results demonstrated that MAb reactive with cell surface domains of an oncogene-encoded protein could directly inhibit tumor growth *in vitro* and *in vivo*.

**3.2.2. b.** MAb induction of Apoptosis

Apoptosis is a phenomenon of developmentally programmed cell death. The mechanisms involved in this process have received much attention recently because of their potential utility in the development of cancer therapy. It has been reported that a MAb against the 52 kDa APO-1 antigen expressed on leukemic cells and human B and T cells completely blocked the proliferation of APO-1 bearing cells *in vitro* by triggering a process of apoptosis. *In vivo*, this anti-APO-1 antibody induced rapid tumor regression of BJAB lymphoma cells growing in nude mice. These findings suggested that antibodies which can induce apoptosis may have beneficial effects on tumor growth *in vivo* (Trauth et al., 1989).

**3.2.3.** MAb as carriers of cytotoxic agents

MAbs have also been utilized as carriers of anti-cancer substances to the tumor cells because of their ability to selectively recognize and bind to the neoplastic cells and bypass normal cells and tissues. This specificity can increase the efficiency of drug delivery and reduce the side effects associated with anti-cancer drugs (Schwartz et al., 1992). Most cell surface receptors are rapidly internalized by the cell. These receptors can be used as targets for MAb for delivery of radioisotopes, immunotoxins, cytotoxic drugs or other agents directly to the malignant cells. Specific cytotoxicity then occurs through local release of the toxic substances and resulting direct action of these potentially cytotoxic substances on MAb coated tumor cells.

3.2.3. a. Radio-immunoconjugates

The principle advantage of radioimmunotherapy is the ability to deliver radiation over a distance of several cell diameters thereby overcoming the obstacles due to antigenic heterogeneity of tumor cells (Goldenberg, 1989). β-emitting isotopes are most commonly chosen for radioimmunotherapy because they can transmit energy over a span of several cell diameters. The radionuclide most commonly used for therapy is $^{131}I$, because of the availability and ease of radiolabeling as well as the emission of high energy β particles. A number of radioimmunotherapy trials have shown objective responses, especially in the treatment of radiosensitive tumors such as lymphomas (Goldenberg et al., 1991). Most clinical trials have utilized $^{131}I$ - labeled MAbs with tumor regression observed in some patients. In a Phase I study using high doses of $^{131}I$-labeled anti-CD37 MAb MB-1 in 10 patients with different grade of non-Hodgkin's lymphoma were evaluated (Press et al., 1989). Five patients showed preferential localization and retention of the MAb at the tumor site. Complete remission was attained in four patients with minimal nonhematologic toxicity.

$^{90}Y$ is the second most commonly used radioisotope which is also a high energy β-emitter with a short half life. Vriesendorp and his colleagues reported that treatment of end-stage Hodgkin's disease using $^{90}Y$-labeled polyclonal anti-ferritin antibody resulted in a complete remissions in 30% of patients (Vriesendorp et al., 1991).

3.2.3. b. Chemo-immunoconjugates

The general advantages of using antibody - conjugated drugs include: 1. the specificity of the conjugates, 2. lowered drug toxicity, 3. increased accumulation of the drug in the tumors and 4. prolongation of the serum half life of the specific drug (Pietersz et al., 1994). The chemotherapeutic agents most commonly used in experimental or clinical trials of immuno-chemotherapy include doxorubicin, N-acetylmelphalan, mitomycin C, neocarzinostatin and methotrexate.

Several studies have demonstrated the potential clinical application of chemo-immunoconjugates. For example, murine MAb A7 which recognizes a human adenocarcinoma antigen was used as carrier for neocarzinostatin (NCS) (Kitamura et al., 1992). Significant tumoricidal effects were observed when the antigen-positive tumor-bearing mice were treated with the A7-NCS conjugate (Kitamura et al., 1993). The toxicity of A7-NCS was five times lower then NCS alone. Another line of evidence comes from Pietersz and his colleagues, using melphalan-anti-CEA in clinical trials of a Phase I/II study of colon cancer. They found that the usual side effects of the drug were absent, the drug could be administered at doses exceeding the maximum tolerated dose established with unconjugated melphalan, large amounts of antibody (in excess of 2 gm) could be given without side effects, partial or complete responses were observed, and one patient with minimal residual was cured by the therapy (Pietersz et al., 1994). These studies demonstrated that MAb can confer more favorable pharmacological

properties on an anticancer drug, making it potentially more useful for cancer chemotherapy.

3.2.3. c. Immunotoxins

The term "immunotoxin" has generally been reserved for conjugates in which the toxic moiety is a ribosomal inhibiting protein. These proteins act enzymatically to inhibit protein synthesis (Olsnes and Sandvig, 1988). A wide range of plant, bacterial and animals toxins have been identified and they can be coupled to MAb for delivery to the tumor cells (Frankel et al., 1986). Many clinical trials have been performed with such conjugates including studies with MAbs linked to the ricin-A chain (Spitler et al., 1987) or the pseudomonas exotoxin (Waldmann, 1991) or to their modified forms (Chaudhary et al., 1989). Different degrees of remissions were observed in these studies.

## 3.3. MAb against adhesion molecules

In recent years, cell-cell , and cell-extracellular matrix adhesion molecules have been used as potential targets for MAb. Antibodies directed to such molecules have been frequently reported to inhibit tumor cell invasion *in vitro*. When tested *in vivo*, some of the antibodies could also inhibit experimental metastases formation. Since the objective of my research project was to use MAb to study the role of cell adhesion in cancer metastasis, a brief summary of the evidence that MAb against adhesion molecules can disrupt the metastatic process is provided.

**3.3.1.** MAb to tumor - endothelial cell adhesion receptors

Tumor cell adhesion to vascular endothelial cells can be mediated by several adhesion molecules expressed on the tumor cell including integrins LFA-1 and VLA-4 which bind to their counter receptors on endothelial cells ICAM-1 and ICAM-2 (for LFA-1) and VCAM-1 (for VLA-4). The evidence suggest that these adhesion molecules play an important role in cancer cell metastasis to specific organs (also see 1.3.2.c).

Lymphocyte function-associated antigen-1 (LFA-1) is a leukocyte integrin which is expressed on lymphocytes (Springer et al., 1987) as well as on some lymphoma cells (Roossien et al., 1989). The LFA-1 ligands ICAM-1 or ICAM-2 are usually expressed on endothelial cells. It has been shown using an *in vitro* invasion model that LFA-1 is important in the invasion of hepatic and fibroblast monolayers by LFA-1 (+) murine lymphoma cells (Roossien et al., 1989). This adhesion molecule was also implicated in invasion of human endothelium *in vitro* by JY lymphoma cells and in liver metastasis of two murine leukemia lines namely the P815 mastocytoma and the EL4 lymphoma (Harning et al., 1993). A murine anti-human LFA-1 ($\alpha$ subunit) MAb inhibited JY lymphoma cell invasion *in vitro*. When assayed *in vivo*, a rat anti-LFA-1 ($\alpha$ subunit) MAb significantly inhibited the development of experimental liver metastases when administered concomitantly with either P815 or EL4 tumor cells.

Lung-specific endothelial cell adhesion molecule-1 (Lu-ECAM-1) is a 90 kDa lectin-like adhesion molecule expressed on endothelia of distinct branches of

the lung blood vessels (Zhu et al., 1991, 1993). It promotes $Ca^{+2}$-dependent adhesion of lung-metastatic B16 melanoma cells. It has been reported that binding of B16 melanoma cell to Lu-ECAM-1 coated plastic surface *in vitro* was blocked by MAb 6D3 to Lu-ECAM-1. This binding was also competitively inhibited by soluble Lu-ECAM-1 (Zhu et al., 1992). Injection of 6D3 MAb into animals after i.v. inoculation of the highly metastatic melanoma cell line, caused a 90% reduction in the number of lung colonies (Zhu et al., 1991). Passive immunization of animals with anti-Lu-ECAM-1 MAb 6D3 or active immunization with purified Lu-ECAM-1 resulted in an anti-Lu-ECAM-1 antibody titer-dependent reduction in the number of lung metastases (Zhu et al., 1992). These studies indicated that anti-adhesion therapy aimed at interfering with the adhesion between blood-borne cancer cells and organ-specific vascular endothelium could potentially be used to prevent the formation of secondary tumor colonies in distant organs.

### 3.3.2. MAb to integrins involved in cell-ECM interactions

In addition to integrins mediating cell-cell adhesion, several antibodies have been produced which can inhibit integrin - mediated adhesion to the ECM.

Integrin $\alpha_5\beta_1$ is a fibronectin receptor which recognizes the RGD site in ECM proteins (Charo et al., 1990). This integrin is expressed on various tumor cells including human breast carcinoma cells (Newton et al., 1995). MAbs specific for the $\alpha_5$ or $\beta_1$ subunits of this integrin could inhibit human breast carcinoma cell attachment to and migration on a fibronectin - coated substratum. These antibodies

also inhibited invasion of tumor cells through an artificial basement membrane. Treatment of human breast carcinoma cells with these MAbs prior to i.v. injection into the athymic nude mice significantly reduced the number of lung colonization (Newton et al., 1995). It was concluded that the inhibitory effect of these antibodies on cancer cell attachment to fibronectin *in vitro* and lung colonization *in vivo* were due to the binding and neutralization of integrin $\alpha_5\beta_1$ function on the tumor cells.

Integrin $\alpha_6\beta_1$ was found to be expressed on the luminal and basalateral sides of the vascular endothelium (Ruiz et al., 1993). It was also detected on the highly metastatic lung-specific cell lines B16/129 melanoma and KLN-205 carcinoma. A MAb EA-1 raised against the $\alpha_6$ chain of $\alpha_6\beta_1$ or $\alpha_6\beta_4$ blocked adhesion of these tumor cells to lung frozen sections *in vitro*, and also inhibited experimental lung metastasis of B16/129 cells when it was injected i.v. into animals before or simultaneously with the tumor cells or when the tumor cells were precoated with EA-1 antibody before injection. Since MAb EA-1 did not interfere with the binding of melanoma cells to laminin fragments (the $\alpha_6\beta_1$ ligand), it was postulated that the antibody recognized a novel ligand - binding domain on the $\alpha_6$ integrins which is involved in cell-cell interaction and in cancer metastasis.

### 3.3.3. MAb directed to CD44

CD44 is an integral membrane glycoprotein initially identified on lymphocytes. This receptor is involved in the adhesion of lymphocytes to

specialized endothelial cells in lymphatic organs and can mediate cell binding mainly to hyaluronate (also see 1.3.1.d.).

Several splice variants of the CD44 molecule with additional inserts in the extracellular domain have been detected on tumor cells (Günthert et al., 1991). It has been reported by Seiter and his colleagues that a MAb directed to a metastasis-specific domain of the rat CD44 molecule namely MAb 1.1ASML (IgG1) retarded growth of the metastatic rat pancreatic adenocarcinoma tumor (BSp73ASML) in the regional draining lymph nodes and inhibited metastasis to the lung (Seiter et al., 1993). This antibody could also efficiently prevent formation of metastasis by a cell line transfected with the metastasis - inducing variant of CD44. The reduction in metastases formation caused by MAb 1.1ASML was not mediated by immune mechanisms as it was shown that preimmunization with MAb 1.1ASML did not reduce metastatic spread. As well, MAb 1.1ASML did not induce significant cytotoxicity towards the BSp73ASML tumor cells in the presence of macrophages, NK-enriched spleen cells and alloantiserum. This study suggested that the antibody interfered with the proliferation of metastasizing tumor cells in the draining lymph node probably by blocking a CD44 - ligand interaction.

High levels of a hematopoietic form of the CD44 molecule have been detected on a human metastatic melanoma cell line SMMU-2 and it was found that the molecules mediated binding to hyaluronic acid *in vitro* (Guo et al., 1994). An anti-human CD44 antibody completely inhibited the binding of the SMMU-2 tumor cells to hyaluronic acid *in vitro*. *In vivo* experiments showed that this

antibody could inhibit the formation of metastases and prolong animal survival. This inhibitory effect was probably due to the blocking of CD44 - mediated adhesion to hyaluronic acid, an interaction which may be necessary for melanoma growth and metastasis.

**3.3.4.** MAb mediated blocking of other cell-substratum interactions

Vollmers and coworkers have produced syngeneic MAbs directed against antigens on B16 melanoma cells. These MAbs blocked the adhesion of melanoma cells to tissue culture dishes, and abolished lung colonization by a highly invasive B16 subline injected i.v. into the animals (Vollmers and Birchmeier, 1983a). *In vitro* these MAbs blocked attachment to poly L-Lysine coated plates of the majority of human melanoma cell lines tested as well as that of murine and human carcinoma, neuroblastoma and glioblastoma cells (Vollmers and Birchmeier, 1983b). Subsequently it was found that the inhibitory effect of this MAb on B-16 melanoma cells was due to the interference with cell adhesion to laminin (Vollmers et al., 1984). A MAb, 14C5 bound an extracellular plasma membrane antigen of SK-BR-3 and MCF-7 human breast cancer cells has been produced by De Potter and his colleagues (De Potter et al., 1994). This MAb bound to a 50 - 90 kDa protein complex expressed on these human breast cancer cells and inhibited cell substrate adhesion of both tumor cell lines *in vitro* to pronectin, osteopontin and vitronectin coated wells. It also delayed the adhesion of MCF-7 and SK-BR-3 cells to precultured embryonic heart fragments (PHF-provided extracellular matrix) and

inhibited the destruction of PHF by MCF-7 cells as well as the invasion of the PHF by SK-BR-3 cells. An immunohistochemical study showed that poorly differentiated, highly invasive breast ductal carcinomas stained extensively with the MAb specifically along plasma membrane extensions. These data indicated that the cell-surface determinant detected by this MAb played a role in cell-substratum adhesion and in the invasion of breast cancer cells.

### 3.3.5. Conclusion

The evidence summarized above indicates that MAb directed to cell surface adhesion molecules mediating tumor - host cell or tumor - host organ ECM interactions necessary for metastases formation can potentially inhibit tumor cell spread into host organs and thereby inhibit the metastatic process. Such antibodies may provide potent immunotherapeutic tools for prevention and treatment of metastasis.

# 4. Lewis lung carcinoma (3LL) and the sublines

## 4.1. Lewis lung carcinoma: origin and metastatic properties

Lewis lung carcinoma (3LL) arose as a spontaneous lung carcinoma in a C57BL/6 mouse (Sugiura and Stock, 1955) and was diagnosed as a poorly differentiated epidermoid carcinoma. It has been maintained thereafter either by animal transplantation or in culture and has been studied extensively in numerous research centers. The tumor is rapidly growing, reaching a size of approximately 18x12x9 mm 14 days after s.c. transplantation and can become extremely hemorrhagic. The lung metastasis can be produced following subcutaneous, intramuscular or intra-footpad injection of dispersed tumor cells (Gorelik et al., 1978).

Spontaneous liver metastases of 3LL parental cells are rare, but have been generated by intrasplenic injection of the cells (Kopper et al., 1982; Paku and Lapis, 1993). Hepatic metastases of 3LL have been reported to occur in hepatic acinar zone 1 regardless of how the cells reach the liver, the diameter of the sinusoids, the concentration of Kupffer cells in each zone and the distribution of ECM in the subendothelial space (Barberá-Guillem et al., 1989).

## 4.2. The sublines H-59 and M-27 of 3LL

Two metastatic sublines of 3LL developed in our laboratory are the lung metastatic subline M-27 and liver metastatic subline H-59 (Fig. 5, page 74). The majority of cells of both tumor variants retain the characteristic morphology of the parent line, with the exception of a higher incidence of giant cells in the liver -

metastatic subline (Brodt, 1986). The growth rates of these two sublines *in vitro* and *in vivo* are similar. Visible tumors are developed by 14 days following the s.c. injection of $10^5$ tumor cells.

M-27 was selected and subcloned from a 3LL pulmonary metastases (Brodt, 1986). Similarly to the parent line cells, M-27 cells form pulmonary metastases only following i.v. or s.c. injection of tumor cells. Lymphatic metastases of M-27 have not to date been observed. M-27 cells do not metastasize to the liver following s.c. or i.v. injection of tumor cells.

H-59, the tumor model used for my study was selected and subcloned from rare 3LL hepatic metastases. When injected s.c. into C57BL/6 mice, tumor H-59 cells metastasizes to the regional lymph nodes draining the tumor site to the mesenteric lymph nodes and to the liver (Brodt, 1986). The ability of H-59 cells to metastasize to the lymph nodes *in vivo* was shown to correlate well with their adhesion to frozen lymph node sections, but not to isolated lymphocytes (Brodt, 1989a). A MAb 12/50 which recognized a 37 kDa molecule on H-59 cells surface significantly reduced tumor cell binding to the frozen lymph nodes sections (Shestowsky et al., 1990). H-59 cells can also metastasize to the lung during the later stages of tumor spread.

The ability of H-59 cells to metastasize to the liver was found to correlate well with their adhesion to hepatocytes *in vitro* (Brodt, 1989b). Two subpopulations of H-59 were selected on the basis of adhesion of H-59 cells to hepatocyte monolayers. The highly adherent cell line was found to be highly

metastatic to the liver, whereas the non-adherent cells either failed give rise to or had a low incidence of hepatic metastases in most of the animal injected (Brodt, 1989b).

### 4.3. Other metastasis-associated properties of H-59 cells

Summarized below are other findings obtained in our own and other laboratories relevant to the metastatic properties of H-59 cells.

#### 4.3.1. Adhesion of H-59 cells to ECM proteins

3LL and its sublines H-59 and M-27 were found to adhere differently to extracellular matrix proteins (Chung et al., 1988). It was shown that the parental cell line 3LL adhered preferentially to fibronectin, M-27 adhered well to fibronectin, and type V collagen, but poorly to laminin, type I, and IV collagen or to heparin sulfate, while H-59 cells were highly adherent to laminin as well as to fibronectin, but did not adhere to heparin sulfate or to any of the collagen types tested (type I, IV and V). Since these three related cell lines with differing metastatic specificities have differences in their abilities to bind to defined matrix molecules, such differences may play a role in the preferential colonization of tumor cells in secondary organs *in vivo*.

**4.3.2.** H-59 cells secrete basement membrane degrading enzymes

H-59 cells produce and secrete several basement membrane - degrading proteinases and they differ both quantitatively and qualitatively from enzymes produced by M-27 cells (Brodt et al., 1992). Namely H-59 cells were found to secrete high levels of a 72 kDa gelatinase (also called gelatinase A, type IV collagenase or MMP2, see 1.3.2.), high levels of cathepsin L and low level of cathepsin B, while M-27 cells only express detectable levels of cathepsin B but produce high levels of uPA (Brodt et al., 1992). Both TIMP-2 (tissue inhibitor of metalloproteinases 2) and the cysteine proteinase inhibitor E-64 completely blocked tumor cell invasion *in vitro* in a matrigel (reconstituted basement membrane) invasion assay and treatment of H-59 cells with E-64 markedly reduced their ability to form liver metastases suggesting that gelatinase A and the cysteine proteinases are involved in a proteolytic cascade mediating tumor cell invasion and early events in liver colonization (Navab et al., 1995) .

**4.3.3.** E-selectin mediates adhesion of H-59 cells to liver sinusoidal endothelial cells

*In vitro* studies have shown that H-59 cells adhered to mouse liver sinusoidal endothelial cells significantly better than M-27 cells (Brodt et al., 1995, manuscript submitted). This adhesion could be significantly enhanced when the endothelial cells were preactivated with recombinant mouse TNFα. An anti-E-selectin MAb reacted with the stimulated endothelial cells and inhibited the

enhancement of H-59 adhesion caused by TNFα activation. Pre-treatment of tumor cells with Bz-α-GalNAC (an inhibitor of O-linked glycosylation) abolished the enhanced adhesion due to TNF-α activation of the endothelial cells implicating a mucin in the adhesion. Furthermore an antibody to E-selectin blocked liver metastases formation by H-59 cells. These results suggested that E-selectin on the hepatic endothelial cell can bind H-59 cells through an O-linked glycoprotein and that this adhesion plays a role in H-59 metastasis to the liver.

**4.3.4.** H-59 cells express higher levels of ganglioside GM2

Gangliosides are sialic-acid-containing glycolipids which are distributed in all mammalian plasma membranes but are most abundant in the brain (Svennerholm, 1963). They are involved in many cell functions such as cellular recognition (Kojima and Hakomori, 1989) and adhesion (Cheresh and Klier, 1986). They are also considered to be tumor-associated markers (Hakomori, 1986). Coulombe and Pelletier found that different patterns of ganglioside were expressed on the surface of 3LL, M-27 and H-59 cells (Coulombe and Pelletier, 1993). No differences were found in the ganglioside patterns expressed on 3LL and M-27 cells derived from s.c. tumors or lung metastases whereas metastatic H-59 cells had a higher GM2 ($II^3$NeuAc-GgOse$_3$Cer) level than cells derived from local s.c. tumor. Interestingly, GM2 was also identified as the predominant ganglioside in livers of C57BL/6 mice. This led to the conclusion that this

glycolipid may be associated with the establishment of liver metastases possibly by mediating cellular adhesion and motility (Kojima and Hakomori, 1991).

**4.3.5.** Other carbohydrate moieties on H-59 cells

The carbohydrate moietics expressed on parent 3LL cells and on the sublines have been analyzed using lectin binding assays (Kahn et al., 1988). It was found that tumor H-59 expressed increased levels of peanut agglutinin (PNA) and soybean agglutinin (SBA) receptors as compare to the primary tumor cells. Since high levels of these receptors could also be detected on hepatocytes, it is possible that these receptors may also involved in liver colonization by H-59 tumor cells.

**4.3.6.** H-59 cells express IGF-1 receptors

The type I insulin - like growth factor (IGF-1) is involved in growth regulation of both normal and malignant cells (Yee et al., 1988) (also see 1.3.3).

Our laboratory has found that the proliferation of H-59 cells could be stimulated by hepatocyte conditioned medium and identified IGF-1 as the mitogenic factor. H-59 cells were found to express significantly higher levels of mRNA transcripts encoding the IGF-1 receptor than M-27 cells (Long et al., 1994). When H-59 cells were transfected with a plasmid vector expressing IGF-1 receptor cDNA in the antisense orientation, the transfectants had a markedly reduced expression of the IGF-1 receptor, lost the ability to respond to IGF-1 *in vitro* and could not form liver metastases *in vivo* (Long et al., 1995). These results

suggested that paracrine growth stimulation by hepatocyte - derived IGF-1 may play a regulatory mechanism in liver metastasis formation by H-59 cells.

### 4.3.7. Conclusion

Together, these findings indicate that several host and tumor - dependent factors are involved in liver metastases of H-59. These include the attachment of tumor cells to ECM components, the degradation of basement membrane elements, different interactions between the tumor cells and liver tissue elements (cells or ECM) mediated by ganglioside as well as intercellular interactions between the tumor cells and SEC mediated by E-selectin. In addition, paracrine growth stimulation by hepatocyte - derived IGF-1 also plays an important role in regulation of H-59 growth in the liver.

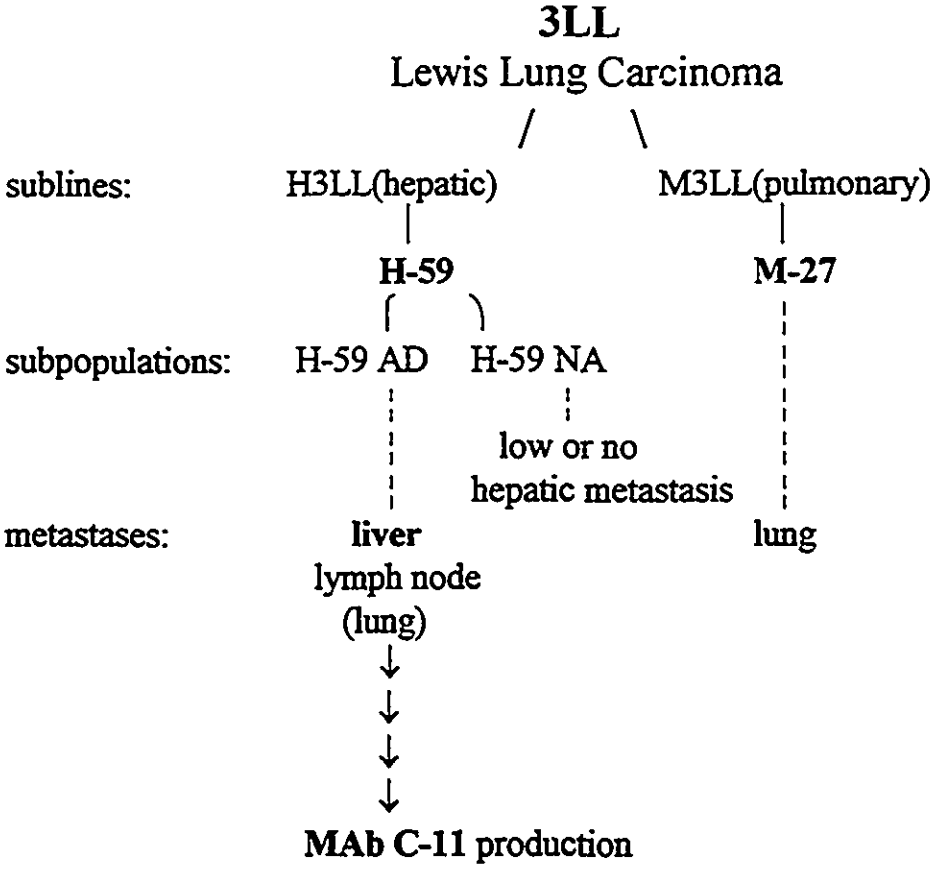



**Figure 5. Lewis lung carcinoma and sublines**

Two metastatic sublines of 3LL used in this study are the liver metastatic subline H-59 and lung metastatic subline M-27. Tumor H-59 cells also metastasize to the regional lymph nodes, and a low incidence of lung metastases has been observed. The H-59 adherent cells (H-59 AD) were selected by adhesion of H-59 cells to hepatocyte monolayers and were highly metastatic to the liver. MAb C-11 was produced by immunization with H-59 AD cells. The H-59 non-adherent cells (H-59 NA) either failed to give rise to or had a low incidence of hepatic metastases.

## 5. Rationale and objectives of this study

Despite progress in recent years in the design of new and potent anti-cancer drugs, the therapeutic options and prognosis for patients diagnosed with liver metastases remain unsatisfactory. The design of more effective therapeutic reagents can be achieved through a better understanding of the biology of the process of liver colonization. Cell adhesion molecules have been widely implicated in malignant dissemination. In particular, cell-cell interaction between tumor cells and organ parenchymal or non-parenchymal cells have been identified as important processes in tumor metastasis.

Liver metastasizing tumor cells are normally retained in the sinusoids of the liver. Since sinusoidal endothelial cells lack a continuous basement membrane and there is no basement membrane around the hepatocytes and furthermore, the hepatocyte microvilli project through endothelial fenestra for ready exposure to sinusoidal content, the tumor cells arrested in the sinusoids can interact directly with the hepatocytes through appropriate adhesion molecule(s) leading to liver metastases formation. Several *in vitro* studies have shown that cell adhesion molecules which mediate tumor cell-hepatocyte adhesion may be involved in liver metastasis. However, the relevance of these molecules to the process of metastasis *in vivo* has not been confirmed by experimental data.

Using the liver metastatic subline H-59 of the Lewis lung carcinoma, we found a positive correlation between the liver - metastasizing potential of H-59 cells *in vivo* and the ability of H-59 cells to adhere to hepatocyte monolayers *in vitro*. In an attempt to identify and characterize the adhesion molecule(s) which were involved in the tumor cell-hepatocyte interaction and to understand the function of this adhesion molecule in liver metastases formation, a MAb (C-11) was produced and used in *in vitro* and *in vivo* experiments. These studies are described in the following chapters. (Chapter II and III).

# CHAPTER II:

## Identification of an Mr 64,000 plasma membrane glycoprotein mediating adhesion of tumor H-59 cells to hepatocytes. *In vitro* event

Wang J, Fallavollita L, Brodt P.

Published in: Cancer Research, 51:3578-3584, 1991

# Chapter II

**Summary:**

Tumor H-59 is a liver-homing variant of Lewis lung carcinoma (3LL). A good correlation has been demonstrated between the liver-metastasizing potential of H-59 cells and their ability to adhere to hepatocytes monolayers *in vitro* (Brodt, 1989). In order to identify the adhesion molecule(s) involved in H-59 cell adhesion to hepatocytes, a MAb, C-11 ($IgG_1$) was produced. In this chapter, the effect of MAb C-11 on cell adhesion *in vitro* was examined. It was found that this MAb was highly specific to hepatocyte-adherent tumor cells as determined by ELISA and indirect immunofluorescence. The antibody as well as its $F(ab)_2$ fragments could block adhesion of H-59 cells to the hepatocyte monolayer when used to pretreat either H-59 cells or hepatocytes. A Western blot analysis revealed that MAb C-11 recognizes a 64 kDa molecule present in H-59 cell lysates or enriched plasma membrane preparations. Under reducing condition, a shift in the migration of this molecule to the 71 kDa region was noted indicating the presence of intrachain disulfide bonds. Pretreatment of the H-59 tumor cell lysate with N-glycosidase F followed by Western blot analysis showed that this enzyme while it did not affect C-11 binding reduced the molecular mass of the C-11 molecule by approximately 43% suggesting that it was heavily glycosylated. The C-11 molecule could also be detected on cultured hepatocytes and on a hepatocyte cell lysate, but was undetectable on spleen cells and thymocytes. Tumor M-27, a lung metastatic subline of 3LL reacted only weakly with MAb C-11.

The results presented in this chapter suggested that a glycoprotein expressed on H-59 cells and hepatocytes mediated the adhesion of tumor H-59 cells to the hepatocytes.

[CANCER RESEARCH 51, 1578–1584, July 1, 1991]



# Identification of an $M_r$ 64,000 Plasma Membrane Glycoprotein Mediating Adhesion of Tumor H-59 Cells to Hepatocytes[1]

Jian Wang,[2] Lucia Fallavollita, and Pnina Brodt[3]

*Department of Surgery, Division of Surgical Research, McGill University, Montreal, Quebec, H3A 1A4, Canada*

## ABSTRACT

Tumor H-59 is a variant of the Lewis lung carcinoma which is metastatic to the liver. In previous studies we have shown that liver metastasis in this tumor model correlates with adhesion *in vitro* to hepatocyte monolayers (Brodt, P., Clin. Exp. Metastasis, *7*: 525–539, 1989). In an attempt to identify the adhesion molecule(s) involved, monoclonal antibodies were produced. One monoclonal antibody (MAb C-11) was highly specific to hepatocyte-adherent tumor cells. The antibody (an IgG1) and $F(ab)_2$ fragments blocked tumor cell attachment to hepatocytes while having no effect on tumor cell adhesion to basement membrane proteins coated onto culture dishes. Western blot analysis of solubilized H-59 plasma membranes or cell lysates showed that the antibody recognizes an $M_r$ 64,000 protein. Treatment with *N*-glycosidase F prior to Western blot analysis revealed that *N*-linked carbohydrate residues constitute approximately 43% of the total weight of this molecule. This glycoprotein is only weakly expressed on tumor M-27, a lung-specific subline of the Lewis lung carcinoma (Brodt, P., Cancer Res., *46*: 2442–2448, 1986), is undetectable in plasma membrane preparations obtained from spleen cells and thymocytes, but can be detected on cultured hepatocytes and in hepatocyte cell lysates. Pretreatment of the hepatocytes with MAb C-11 also resulted in inhibition of tumor cell adhesion. These results suggest that this glycoprotein mediates the attachment of H-59 cells to hepatocytes.

## INTRODUCTION

The host-tumor interactions which regulate the site selectivity of metastasis have been the subject of numerous investigations and much debate in the past century (1, 2). Evidence derived mainly from experimental tumor models and supported by clinical observations indicates that multiple factors, both specific and nonspecific, determine the ultimate site of growth of disseminated tumor cells. Thus, while the lymphatic and vascular drainage of the primary site initially determine tumor cell access to secondary organs, subsequent specific interactions between tumor cells and the target organ vascular endothelium (3–5), extracellular matrix (6–9), and stromal and parenchymal cells (10–12) are thought to regulate tumor cell potential to form metastases (for review, see Refs. 2 and 13). These specific interactions may involve tumor cell adhesion molecules which recognize organ-specific determinants on host cells and matrix and/or soluble mediators present in the organ microenvironment (14–16).

The liver is the primary site of metastasis for several common human malignancies (*e.g.*, carcinomas of the gastrointestinal tract) (17). Yet, the mechanisms regulating liver colonization by tumor cells are not fully understood. A paucity of information is particularly noted in regard to neoplasms of epithelial origin because few experimental models of liver-homing carcinomas are available for study.

In previous studies we found that the potential of tumors H-59 and M-27, two sublines of the Lewis lung carcinoma with different organ specificities to metastasize to the liver, correlated with their adhesion to primary cultures of hepatocytes (18, 19). H-59 cells were subsequently fractionated into two subpopulations with high and low potentials to colonize the liver on the basis of their differential ability to adhere to hepatocyte monolayers (19). In the present investigation an MAb[4] specific to a highly adherent and metastatic subpopulation of tumor H-59 was produced and used to characterize the adhesion molecule involved.

## MATERIALS AND METHODS

### Mice

C57BL/6 female mice (7–12 weeks old) were used for immunization and (C57BL/6 × BALB/c)F1 were used for production and maintenance of hybridoma ascites. The animals were purchased from Charles River Canada (Montreal, Quebec, Canada).

### Cell Lines

The origin and metastatic and adhesive properties of sublines H-59 and M-27 of the Lewis lung carcinoma have been described previously (6,18,19). The tumors were maintained *in vivo* by s.c. implantation of liver (H-59) and lung (M-27) metastases derived from tumor-bearing mice, into new recipient animals.

Single-cell suspensions of the tumors were obtained by enzymatic digestion of the solid tumors in a 0.02% trypsin solution in PBS-EDTA as we described previously (18). Where required, tumor cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum (Gibco, Burlington, Ontario, Canada), 0.01 M *N*-2-hydroxyethylpiperazine-*N*-ethanesulfonic acid, and $2 \times 10^{-3}$ M glutamine. Tumor cells were cultured for 1–2 weeks prior to use in the adhesion assays or prior to preparation of plasma membranes and cell lysates.

Fibroblast line 3T6 was obtained courtesy of Dr. W. Lapp (Dept. of Physiology, McGill University, Montreal, Quebec, Canada) and maintained in RPMI-1640 supplemented with 10% fetal calf serum, 0.01 M *N*-2 hydroxyethyl piperazine-*N*-ethanesulfonic acid, and $2 \times 10^{-3}$ M glutamine. All cells were cultured at 37°C in a 5% $CO_2$ incubator and fed once or twice weekly as required. Monolayers were dispersed by incubation with enzyme-free PBS-EDTA for 5–10 min at 37°C (18).

Monolayers of splenic lymphocytes in microtiter well plates were prepared using poly-L-lysine as previously described (20).

### Adhesion Assay

Primary mouse hepatocyte cultures were prepared by collagenase perfusion (21). The culture conditions and adhesion assay were described in detail elsewhere (19). Nonadherent cells (H-59NA) were harvested by repeated washing of the hepatocyte monolayer with PBS. Adherent cells (H-59AD) were eluted off the monolayers by incubation in PBS-EDTA for 10 min at 37°C. Treatment of the tumor cells with

Received 2/7/91; accepted 4/17/91.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by a grant from the Medical Research Council of Canada.

[2] Recipient of a graduate student award from the Faculty of Medicine and the Royal Victoria Hospital Research Institute.

[3] To whom requests for reprints should be addressed, at Department of Surgery, Division of Surgical Research, McGill University, 740 Docteur Penfield Avenue, Montreal, Quebec, H3A 1A4, Canada.

[4] The abbreviations used are: MAb, monoclonal antibody; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; PM, plasma membrane; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CAM, cell adhesion molecule.

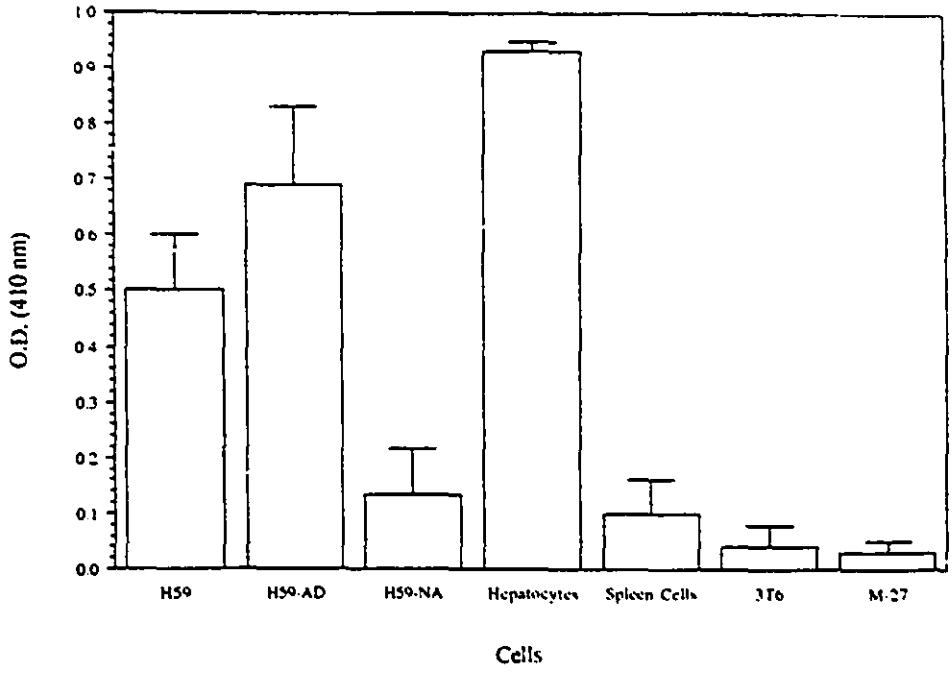



Fig. 1. Antibody C-11 recognizes a subpopulation of tumor H-59 selected for adhesion to hepatocytes. Target cells were seeded into 96-well plates at a density of $5 \times 10^4$ cells/well. ELISA tests were performed on cell monolayers 48 h later. *Columns*, means (*bars*, ±SD) of 4 assays. O.D., absorbance.

tunicamycin and various enzymes prior to the adhesion assay was carried out as previously described (22).

### Adhesion to Matrigel-coated Wells

Matrigel (a kind gift of Dr. H. Kleinman, National Institutes of Health, Bethesda, MD) was added to 24-well plates at a concentration of 1 mg/ml and allowed to dry in a laminar flow hood overnight (23). To each well $5 \times 10^4$ $^{51}$Cr-labeled tumor cells were added and then incubated at 37°C for the specified time intervals. Adhesion was quantitated as described above for tumor adhesion to hepatocytes.

### Production of Monoclonal Antibodies

**Immunization.** The method used to generate MAbs specific for H-59AD cells has been described in detail elsewhere (24). Tolerance to cell surface determinants expressed on H-59NA cells was induced in syngeneic C57BL/6 mice by i.p. injection of $10^7$ γ-irradiated (10,000 rads) H-59NA cells, followed 3 days later by an injection of cyclophosphamide (Procytox; Horner, Montreal, Quebec, Canada) as described before (22). Immunization with H-59AD cells followed, using injections of $10^7$ γ-irradiated cells on alternate weeks. Three days following the third inoculation the animals were sacrificed and their spleens removed for fusion.

**Fusion Procedure and Screening.** Myeloma line SP2/0-Ag 14 (obtained courtesy of Dr. A. Sullivan, McGill Cancer Centre; see Ref. 25) was used for the fusions. The fusion procedure was that previously described (22). Hybridoma supernatants were screened by ELISA (25). Hybridomas which secreted antibodies of interest were cloned by the limiting dilution method.

**Isotyping of the Antibody.** Antibodies were isotyped using the ELISA. A mouse monoclonal subisotyping kit obtained from HyClone Laboratories (Logan, UT) was used according to the instructions of the manufacturer.

**Immunofluorescence Analysis.** Indirect immunofluorescence labeling was carried out as we described in detail elsewhere (22).

**Control Antibodies.** The following antibodies were also used: (*a*) MAb 12/50 (an IgG1) was produced in our laboratory (22). The antibody reacts with tumor H-59 but does not discriminate between adherent and nonadherent cells; (*b*) MAb CL9002 (an IgG3) was obtained from Cedarlane Laboratories (Hornby, Ontario, Canada). It is specific for the H-2D$^b$ determinant which is expressed on tumor H-59; (*c*) MAb 01MLEUK10 (an IgG2a) which reacts with the Ly15.2 (LFA-1) determinant of leukocytes was obtained from Cedarlane Laboratories. Rabbit antisera to BSA was from Cappel (Organon Teknika, Scarborough, Ontario, Canada).

**Purification of Antibodies.** MAbs C-11 and 12/50 were purified by affinity chromatography with Affi-Gel protein A Monoclonal Antibody Purification System (MAPS II; Bio-Rad Laboratories, Mississauga, Ontario, Canada) according to the instructions of the manufacturer.

**Preparation of F(ab)$_2$ Fragments.** F(ab)$_2$ fragments of antibody C-11 were prepared using immobilized pepsin (Pierce, Rockford, IL) according to the instructions of the manufacturer. The Fc fragments were separated on a protein A-agarose affinity columns (see above) and the F(ab)$_2$ fragments further purified by high performance liquid chromatography (Waters; Protein Pak 300 SW; column, 0.75 × 30 cm), using 0.1 M sodium phosphate buffer, pH 6.5, and a flow rate of 0.5 ml/min. The absorbance was monitored at 254 nm (absorbance detector model 441), and the peak fractions were pooled, dialyzed, and concentrated. Purity of the fragments was confirmed by SDS-PAGE as described below.

**Preparation of Cell Lysates.** Cultured cells were harvested and lysed in a solution of 1% Triton X-100 containing 20 mM *N*-2 hydroxyethyl

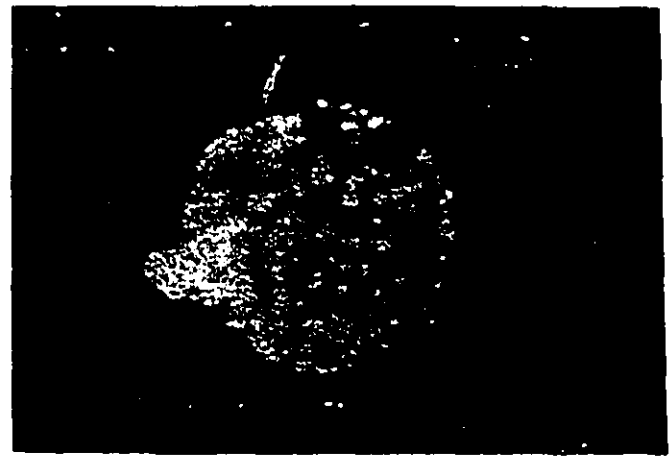


Fig. 2. Indirect immunofluorescence labeling of H-59 cells with MAb C-11. Viable H-59 cells ($2 \times 10^6$ in 0.1 ml medium) were incubated with a 1:20 dilution of C-11 ascites for 1 h at 4°C. Fluorescein isothiocyanate-conjugated goat anti-mouse IgG was used as the second antibody. (× 4500).

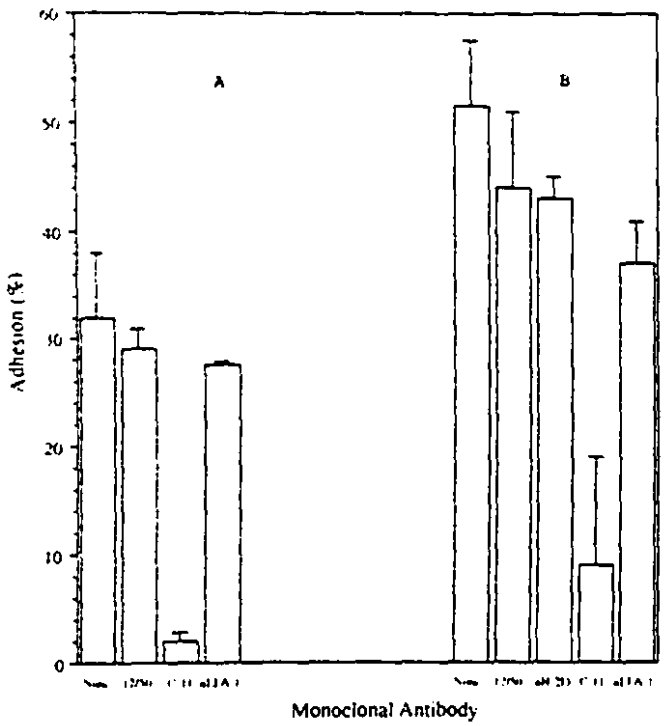



Fig. 3. Antibody C-11 blocks the adhesion of tumor H-59 to hepatocytes. The adhesion of nonfractionated H-59 cells (*A*) and of the adherent tumor cells H-59AD (*B*) was measured following tumor cell incubation with ascites fluid (1:10) of antibodies C-11 and 12/50. MAb CL9002 to the H-2D$^b$ determinant was partially purified ascites fluid diluted 1:100 (this dilution was determined by ELISA to result in a level of antibody binding comparable to that of C-11 ascites fluid at the dilution used). MAb 01MLEUK10 to LFA-1 was used at a dilution of 1:25, at which maximal specific inhibition was seen. Cells ($10^6$ in 0.2 ml) were incubated with the antibodies for 1 h at 4°C and then washed 4 times with RPMI to remove unbound antibodies. Adhesion to hepatocytes was measured as described in "Materials and Methods." *Columns*, means (*bars*, SD) of 3 experiments.

piperazine-*N*-ethanesulfonic acid, 5 mM $MgCl_2$, 2 mM phenylmethysulfonyl fluoride, 0.5 μg/ml leupeptin (Sigma) and 0.7 μg/ml pepstatin (Sigma). The lysate was cleared by centrifugation at 16,000 × *g* for 10 min, and aliquots were prepared and stored at −20°C.

Isolation of PM. PMs were prepared from cultured H-59 and M-27 cells by a modification of the method described by Monneron and d'Alayer (26) as we described in detail elsewhere (22).

Gel Electrophoresis. Prior to analysis by SDS-PAGE, PM preparations were solubilized with 1% Triton X-100 and centrifuged for 60 min at 122,000 × *g* in a Beckman ultracentrifuge to remove aggregates. Solubilized PM or cell lysate samples were separated on 7.5 or 10%

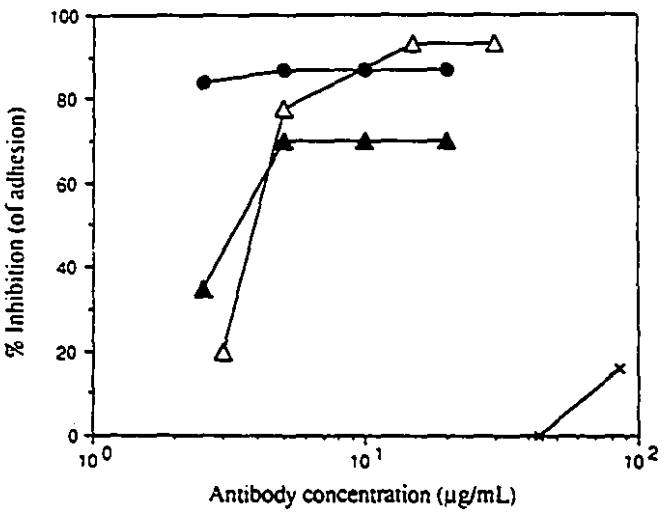



Fig. 5. Inhibition of tumor cell adhesion to hepatocytes by F(ab)$_2$ fragments of antibody C-11. One million H-59 cells were incubated for 1 h at 4°C with 0.2 ml of RPMI containing the specified concentration of purified antibody. Cells incubated with F(ab)$_2$ were added to hepatocytes monolayers either directly (Δ) or following extensive washing to remove unbound antibody (▲). The adhesion assays were carried out as described. Cells treated with whole antibody C-11 (●) or with MAb 12/50 (x) were also washed prior to the adhesion assay. Results are based on triplicate samples. SD, 2–8% of the means.

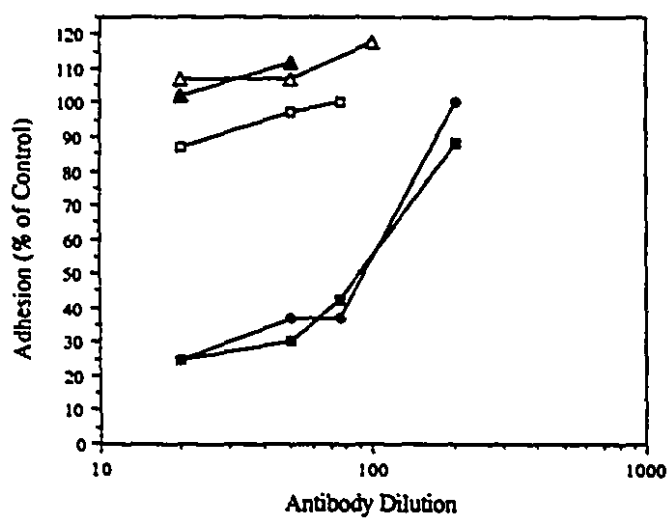



Fig. 4. Pretreatment of hepatocytes or H-59 cells with MAb C-11 inhibits cell-cell adhesion. Prior to the adhesion assay, MAb C-11 ascites (●) or a control ascites produced with myeloma SP2/0-Ag 14 (□) were added at the dilutions indicated (reciprocal of dilutions shown) to 48-h-old hepatocyte cultures and incubated for 1 h at 4°C. Unbound antibody was removed by washing. Hepatocytes incubated with PBS were used as controls. Pretreatment of H-59 cells with C-11 (■) or with the control ascites (□) was carried out under similar conditions. As an added control the effect of MAb C-11 on the adhesion of H-59 cells to culture dishes coated with 1 mg/ml Matrigel (Δ) or 10 mg/ml BSA (▲) were also tested. Incubation was for 60 min at 37°C. Unbound cells were removed by washing. Adhesion of untreated cells to Matrigel-coated dishes (not shown) was 30%, while adhesion to BSA-coated dishes was 2.4%.

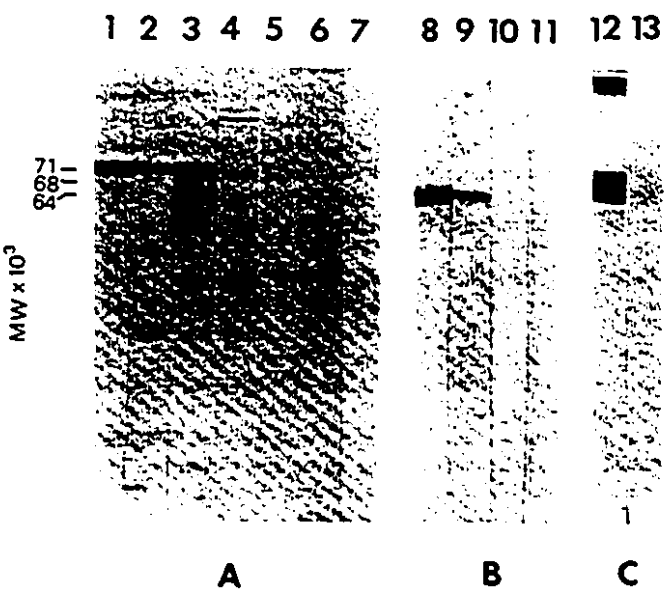



Fig. 6. Western blot analysis with MAb C-11 (*A* and *B*) and anti-BSA (*C*). *A*, results of analyses carried out under reducing condition. *Lane 1*, H-59 lysate; *lane 2*, H-59 solubilized plasma membrane; *lane 3*, hepatocyte lysate; *lane 4*, M-27 lysate; *lane 5*, M-27 plasma membrane; *lane 6*, thymocytes lysate; *lane 7*, spleen cells lysate. *B*, nonreducing conditions. *Lane 8*, H-59 lysate; *lane 9*, M-27 lysate; *lane 10*, H-59 lysate blotted with normal mouse IgG; *lane 11*, H-59 lysate blotted with SP2/0-Ag 14 ascites. *C*, purified BSA blotted with antibody to BSA (*lane 12*) and MAb C-11 (*lane 13*). Proteins were resolved on a 10% SDS-PAGE.

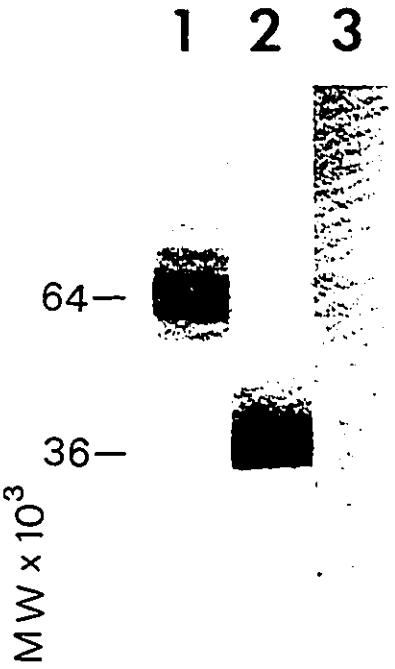



Fig. 7. Western blot analysis of H-59 lysate digested with *N*-glycosidase F. *Lane 1*, untreated lysate; *lane 2*, lysate treated with *N*-glycosidase F; *lane 3*, lysate treated with pronase. The lysate was resolved on 10% polyacrylamide gels under nonreducing conditions.

polyacrylamide gels as specified, using the method of King and Laemmli (27).

**Western Blot Analysis.** The electrophoretic transfer of proteins onto nitrocellulose paper (0.2 μm; Schleicher and Schuell, Montreal, Quebec, Canada) and the subsequent analyses with MAb C-11 were performed as described by Towbin *et al.* (28). After the transfer, the nitrocellulose strips were first incubated for 18 h at 4°C with a solution of 5% skim milk and 3% BSA to block nonspecific binding sites and then for 18 h with C-11 ascites diluted 1:25 in PBS. Alkaline phosphatase-conjugated goat anti-mouse immunoglobulin (Jackson Immuno Research Laboratory) at a dilution of 1:5000 was used as the second antibody and nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (BCIP), (Bio-Can, Toronto, Ontario, Canada) was used as substrate.

**Enzyme Treatment.** In some experiments the cell lysate was subjected to enzyme digestion with either *N*-glycosidase F (*N*-glycanase; Genzyme, Boston, MA) or with pronase (from *Streptomyces griseus*; Boehringer-Mannheim, Penzberg, West Germany) prior to the Western blot analysis. Cell lysate samples were prepared according to the instructions of the manufacturer, and 25 μg of protein was treated with 0.2–1 unit of *N*-glycanase or 0.35–1.75 units of pronase for 18 h at 37°C (29). Control samples were incubated without enzyme.

**Immunoaffinity Chromatography.** Five mg of purified antibody was coupled to Affi-Gel Hz hydrazide gel (Bio-Rad) according to the instructions of the manufacturer. To remove plasma membrane proteins which react nonspecifically with IgG, a second column was prepared using 8 mg of affinity-purified mouse IgG (Jackson Immuno Research Laboratories)). PM preparations were solubilized with Triton X-100, and 2 mg protein in PBS, pH 7.4, containing 0.1% Triton X-100 was applied to the IgG column. The column was washed with 0.5–0.25 M NaCl containing 0.05% Triton X-100 and the unbound protein applied to the C-11 column at a rate of 0.15 ml/min. The C-11-bound protein was eluted at the same flow rate with 0.2 M glycine-HCl, pH 2.5. The pH was readjusted with Tris-HCl, pH 9.0, and the peak fractions (determined on the basis of absorbance at 280 nm) were pooled and concentrated in an Amicon ultrafiltration cell. The eluate was separated by SDS-PAGE, and the protein band was silver stained using Bio-Rad reagents according to the instructions of the manufacturer.

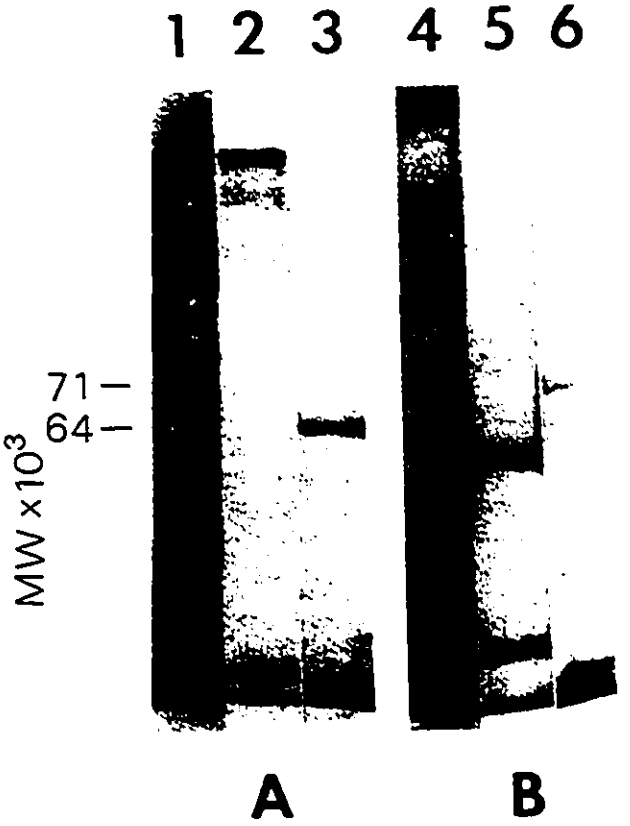



Fig. 8. SDS-PAGE of solubilized H-59 plasma membranes purified by immunoaffinity chromatography on C-11 coupled agarose gels. Two mg of Triton X-100 solubilized plasma membrane proteins was first loaded onto the IgG column and the unbound protein passed on the C-11 column. Eluted proteins were resolved on a 7.5% polyacrylamide gel under (*A*) nonreducing conditions and (*B*) reducing conditions. *Lanes 1* and *4*, solubilized unfractionated plasma membrane; *lanes 2* and *5*, proteins eluted from the IgG; *lane 3* and *6* proteins eluted from the C-11 column.

Table 1 *Effect of enzyme or tunicamycin treatment on the adhesion of tumor H-59 to hepatocytes*

Enzyme treatment was carried out as described previously (22). Treatment with tunicamycin (3 mg/ml) was for 48 h (7). Cell viability after enzyme treatment was ≥90% and after the tunicamycin treatment ≥70%. Cell concentrations were readjusted to deliver the same number of viable cells in each treatment group. Adhesion assay was carried out as described (19). Results are means ± SD of triplicates.

| Treatment | Adhesion (% of control) |
|---|---|
| β-galactosidase | 100 ± 7 |
| Neuraminidase | 100 ± 2 |
| Neuraminidase + β-galactosidase | 50 ± 10 |
| Exoglycosidases[a] | 30 ± 1.9 |
| Tunicamycin | 30 ± 1.5 |
| Pronase | 6 ± 2.2 |

[a] A cocktail of *C. lampas* exoglycosidases was used containing the following enzymes: α-*N*-acetylgalactosaminidase, β-*N*-acetylhexosaminidase, α-L-fucosidase, β-galactosidase, and α-mannosidase in a buffer of 8 mM $Na_2HPO_4$, 1 mM $KH_2PO_4$, and 0.1 M sodium citrate (pH 6.2).

## RESULTS

Hybridomas were screened by ELISA on monolayers of H-59AD, H-59NA, and M-27 cells (19, 22). Hybridoma C-11 was subsequently cloned and selected for further study because it was highly specific for the adherent subpopulation of tumor H-59 as shown in Fig. 1. The antibody reacted poorly with tumor M-27, 3T6 fibroblasts, and normal syngeneic splenocytes but gave a strongly positive reaction with primary cultures of hepatocytes (Fig. 1). Immunofluorescence analysis with fluorescein isothiocyanate-conjugated goat anti-mouse IgG confirmed the specificity of the antibody for the adherent subpopulation as 60% of the adherent cells, but only 12% of the nonadherent cells were positively labeled. A tumor H-59 cell labeled with the antibody is shown in Fig. 2.

The ability of antibody C-11 to block tumor cell adhesion to hepatocyte monolayers *in vitro* was subsequently tested. Tumor cells were pretreated with antibody C-11 or with three other MAbs with different antigenic specificities and their attachment to hepatocyte monolayers were compared to that of untreated cells. Results of these experiments, shown in Fig. 3, demonstrate that antibody C-11 inhibited tumor binding significantly better than any of the other antibodies tested (87% inhibition). MAb 12/50 and a MAb directed against the H-2D$^b$ determinant failed to inhibit binding (Figs. 3 and 5), while a MAb specific for the LFA-1 molecule (30) had a minor inhibitory effect on the adhesion of H-59AD cells (maximal inhibition, 29%).

Adhesion could also be inhibited by pretreatment of the hepatocytes with MAb C-11 as shown in Fig. 4. Normal mouse IgG and a control ascites did not significantly block tumor cell adhesion to hepatocytes, while antibody C-11 failed to block adhesion of H-59 cells to Matrigel or BSA-coated culture dishes (Fig. 4). Adhesion to laminin and type IV collagen-coated dishes was also not affected by pretreatment with MAb C-11 (not shown).

F(ab)$_2$ fragments of antibody C-11 also inhibited tumor cell adhesion to the hepatocytes as shown in Fig. 5. Treatment with F(ab)$_2$ fragments was most effective when they were added to the hepatocyte cultures together with the tumor cells.

Western blot analysis as shown in Fig. 6 revealed that the antibody detected an $M_r$ 64,000 protein also found in hepatocyte cell lysates. Under reducing condition this protein migrated to the $M_r$ 71,000 region. There was no detectable band in cell lysates obtained from spleen cells and thymocytes, while a weak reaction was detected with tumor M-27. The protein did not react with either normal mouse IgG or ascites fluid obtained with myeloma SP2/0-Ag 14 (Fig. 6*B*). MAb C-11 gave no reaction with purified BSA (Fig. 6*C*), while antibodies to BSA did not react with a cell lysate of tumor H-59 (not shown).

Treatment of the cell lysate with *N*-glycosidase F did not affect antibody binding and revealed that *N*-linked carbohydrates constitute approximately 43% of the total weight of this molecule (Fig. 7). Pretreatment with pronase abolished antibody binding.

Immunoaffinity chromatography using solubilized H-59 membrane and immobilized C-11 antibody was subsequently used to purify this glycoprotein. Results shown in Fig. 8 confirmed the Western blot finding and showed that the antibody recognized a single $M_r$ 64,000 plasma membrane protein.

To test whether carbohydrate residues played a role in tumor cell adhesion to the hepatocytes, the cells were treated with either tunicamycin or with several exoglycosidases prior to the adhesion assay. Results shown in Table 1 demonstrate that tumor cell adhesion to hepatocytes was significantly reduced following treatment with tunicamycin or *Charonia lampas* exoglycosidases and to a lesser extent by a combination of neuraminidase and β-galactosidase. Treatment with either β-galactosidase or neuraminidase alone had no effect.

## DISCUSSION

CAMs are now known to play a central role in the process of cancer dissemination by mediating cell-cell and cell-substratum interactions which are essential for tumor cell invasion and proliferation (2). While the potential involvement of CAMs in the organ site specificity displayed by some metastatic cancers has been postulated (1, 2, 13), few tumor adhesion molecules which are involved in the homing of cancer cells, carcinomas in particular, have actually been identified (12, 31).

The present study was prompted by our earlier observation that a subpopulation of tumor H-59 which was highly metastatic to the liver *in vivo* had a significantly elevated level of adhesion to hepatocyte monolayers *in vitro* as compared to nonmetastatic cells. This was in agreement with other reports based on studies of lymphoma and lymphosarcoma lines which also linked hepatic metastases formation with tumor potential to adhere to hepatocytes (32) and/or invade hepatocyte monolayers *in vitro* (33).

In an attempt to identify the adhesion receptors involved in tumor-hepatocyte adhesion in this tumor model, a MAb specific for the liver-adherent fraction of tumor H-59 (H-59AD) was produced. This antibody (C-11) inhibited the adhesion to hepatocytes in a specific manner and had little effect on the attachment of H-59 cells to BSA or extracellular matrix protein-coated dishes or primary cultures of liver sinusoidal endothelial cells.[5] Moreover, adhesion to hepatocytes could not be blocked by pretreatment of the tumor cells with either normal mouse IgG or two control IgG MAbs. A third MAb to the LFA-1 determinant inhibited adhesion only minimally (maximal inhibition, 29%). These findings coupled with our observation that the antibody bound poorly to the lung-specific line M-27 or to H-59NA cells suggest that C-11 recognizes a cell surface molecule directly involved in the adhesion of H-59 cells to hepatocytes.

Western blot analysis revealed that C-11 recognizes an $M_r$ 64,000 plasma membrane glycoprotein which under reducing conditions shifted to the $M_r$ 71,000 region, indicating the presence of intrachain disulfide bonds. *N*-linked carbohydrate residues were found to constitute approximately 43% of the weight of this molecule. Our data suggest that the *N*-linked carbohydrate moieties are not essential for MAb C-11 recognition but may play a functional role in adhesion because the enzymatic removal of cell surface carbohydrates significantly reduced tumor adhesion to hepatocytes. These results, however, do not rule out the possible involvement of glycoconjugates present on cell surface molecules other than the C-11 antigen.

The C-11 determinant is also expressed on hepatocytes as shown by ELISA and Western blot analysis. Blocking experiments suggested that cell-cell adhesion in this model requires participation of the molecules expressed on both cell types. Whether this interaction involves a homophilic cadherin-like recognition process (34) or is mediated by different receptor and ligand structures expressed on both cell types (heterotypic

[5] P. Brodt and O. Morin. The adhesion of tumor H-59 cells to hepatic sinusoidal endothelial cells and to hepatocytes are mediated by different adhesion receptors, manuscript in preparation.

adhesion) remains to be determined.

Several classes of adhesion receptors which mediate cell-cell adhesion in developmental processes, inflammation, host immune responses, and possibly metastasis have been described. Best characterized among them are (*a*) the integrin family of adhesion receptors, in particular the $\beta_2$ subgroup which plays a critical role in leukocyte adhesion (for review, see Ref. 35), (*b*) the adhesion receptors belonging to the IgG superfamily, also implicated in blood cell interactions (36), (*c*) the $Ca^{2+}$-dependent cadherins which mediate homophilic adhesion and play a regulatory role during development (37), and (*d*) the LEC CAMS which are expressed on a variety of cell types and mediate lectin-like adhesive cell-cell interactions which can be blocked by specific sugars. This group includes several adhesion receptors expressed on leukocytes (LECAM 1) and endothelial cells (ELAM 1 or LECAM 2) which play a role in leukocyte traffic and homing (38, 39).

Our data suggest that the adhesion receptor recognized by C-11 does not belong to one of the leukocyte adhesion systems previously described (35) because MAb C-11 did not react with lymphocytes and thymocytes in ELISA and no reaction could be detected when cell lysates of spleen cells and thymocytes were blotted with the antibody. In recent preliminary studies (not shown), we found that a MAb directed to the mouse $\beta_2$-integrin chain (antibody M18.2.a.8 obtained courtesy of Dr. T. Springer, Boston, MA) failed to block adhesion of H-59 cells to hepatocytes. In addition (as we have shown), antibodies to LFA-1 had a minimal inhibitory effect on adhesion of H-59 cells to hepatocytes. Thus, it appears that the adhesion in the present model system is mediated by mechanisms different from the $\beta_2$-integrin-dependent adhesion described recently for liver-metastasizing lymphoma lines (40). The mechanism responsible for the low level of inhibition observed when H-59AD cells were treated with antibodies to LFA-1 is unclear. One possible explanation may be that H-59 cell adhesion to hepatocytes increases expression of the LFA-1 receptor on the tumor cells and that once expressed these molecules play a secondary role in the adhesion.

The molecular weight range of the C-11 antigen and its relatively high degree of *N*-linked glycosylation exclude many of the above-mentioned CAM families including the integrin subunits, the cadherins, and some of the larger adhesion receptors of the IgG superfamily. Because our findings suggest that carbohydrates are involved in the present adhesion system, it is conceivable that a hepatocyte lectin which could be abnormally expressed on tumor H-59 cells may be involved in the adhesion. This is supported by our previous results of an *in situ* lectin-binding study. In this study it was found that liver metastases of tumor H-59 expressed increased levels of peanut agglutinin receptors (as compared to the primary s.c. tumor) and that high levels of these receptors could also be detected on hepatocytes (41).

Tumor cell surface oligosaccharides have previously been shown to play a role in metastasis (42, 43) and to mediate adhesion to hepatocytes (12). Endogenous tumor lectins have also been been implicated in tumor cell adhesion and metastasis (44). One of the better characterized liver lectins, namely, the asialoglycoprotein receptor, has previously been shown to mediate cell-cell adhesion to hepatocytes as well as to Kupffer cells and liver sinusoidal endothelial cells (45). Although the participation of this lectin in the present adhesion system cannot be entirely ruled out, the results of the enzyme studies argue that it is probably not the major hepatocyte receptor involved, because treatment of the tumor cells with neuraminidase or $\beta$-galactosidase failed to modify adhesion, while treatment with both enzymes only reduced adhesion by 50%.

The role of tumor cell adhesion to hepatocytes in the process of liver colonization is still unknown. Sargent *et al.* (15) have recently shown that tumor-hepatocyte contact is required for a growth stimulatory effect exerted by a diffusible hepatocyte factor on a liver-metastasizing subline of melanoma B-16. Preliminary data in our laboratory also indicated that, under conditions which permit cell-cell contact, H-59 cells cocultured with hepatocytes show an increased uptake of [$^3$H]thymidine as compared to controls.* The nature of the growth factor(s) and the relationship between adhesion and proliferation in the present tumor model are the subject of present investigations in our laboratory and should be facilitated by the availability of antibody C-11.

Recently, we reported the inhibition of liver colonization by administration *in vivo* of MAb C-11 (46). Similar findings were also reported by McGuire *et al.* (47). Although the mechanism of action of the C-11 antibody *in vivo* remains to be elucidated, our data and studies by other laboratories (48) suggest that inhibition of cellular adhesion *in vivo* by specific reagents, in conjunction with existing treatment protocols, will provide a promising new avenue for therapeutic intervention in the process of cancer metastasis.

## REFERENCES


1. Hart, I. R. Seed and soil revisited. Mechanisms of site specific metastasis. Cancer Metastasis Rev., *1:* 5–16, 1982.
2. Nicolson, G. L. Organ specificity of tumor metastasis: role of preferential adhesion, invasion and growth of malignant cells at specific secondary sites. Cancer Metastasis Rev., *7:* 143–188, 1988.
3. Pauli, B. V., and Lee, C-L. Organ preference of metastasis. The role of organ-specifically modulated endothelial cells. Lab. Invest., *58:* 379–387, 1988.
4. Lichtner, R. B., Belloni P. M., and Nicolson, G. L. Differential adhesion of metastatic rat mammary carcinoma cells to organ-derived microvessel endothelial cells and subendothelial matrix. Exp. Cell Biol., *57:* 146–152, 1989.
5. Weiss, L., Orr, F. W., and Honn, K. V. Interactions between cancer cells and the microvasculature: a rate-regulator for metastasis. Clin. Exp. Metastasis, *7:* 127–167, 1989.
6. Chung, D. C., Zetter, B. R., and Brodt, P. Lewis lung carcinoma variants with differing metastatic specificities adhere preferentially to different defined extracellular matrix molecules. Invasion Metastasis, *8:* 103–117, 1988.
7. Brodt, P., Fallavollita, L., Sawka R. J., Shibata, P., Nip, J., Kim, U., and Shibata, H. Tumor cell adhesion to frozen lymph node sections—a correlate of lymphatic metastasis in breast carcinoma models of human and rat origin. Breast Cancer Res. Treat., *17:* 109–120, 1990.
8. Ruoslahti, E., and Giancotti, F. G. Integrins and tumor cell dissemination. Cancer Cells, *1:* 119–126, 1989.
9. Doerr, R., Zvibel, I., Chiuten, D., D'Olimpio, J., and Reid, L. M. Clonal growth of tumors on tissue-specific biomatrices and correlation with organ site specificity of metastases. Cancer Res., *49:* 384–392, 1989.
10. Middelkoop, O. P., van Bavel, P., Calafat, J., and Roos, E. Hepatocyte surface molecule involved in the adhesion of TA3 mammary carcinoma cells to rat hepatocyte cultures. Cancer Res., *45:* 3825–3835, 1985.
11. La Riviere, G., Schipper C. A., Collard, J. G., and Roos, E. Invasiveness in hepatocyte and fibroblast monolayers and metastatic potential of T-cell hybridomas in mice. Cancer Res., *48:* 3405–3410, 1988.
12. Schlepper-Schafer, J., and Springer, G. F. Carcinoma autoantigens T and Tn and their cleavage products interact with Gal/GalNAc-specific receptors on rat Kupffer cells and hepatocytes. Biochim. Biophys. Acta, *1013:* 266–272, 1989.
13. Zetter, B. R. The cellular basis of site specific tumor metastasis. N. Engl. J. Med., *322:* 605–612, 1990.
14. Price, J. E., Tarin, D., and Fidler, I. J. Influence of organ microenvironment on pigmentation of a metastatic murine melanoma. Cancer Res., *48:* 2258–2264, 1988.
15. Sargent, N. S. E., Oestreicher, M., Haidvogl, H., Madnick, H. M., and Burger, M. M. Growth regulation of cancer metastases by their host organ. Proc. Natl. Acad. Sci. USA, *85:* 7251–7255, 1988.
16. Chackal-Roy, M., Niemeyer, C., Moore, M., and Zetter, B. R. Stimulation of human prostatic carcinoma cell growth by factors present in human bone


* Unpublished observation.

marrow. J. Clin. Invest., *84:* 43–50, 1989.
17. Platz, C. E. The staging and pathology of colonic carcinoma and adenomas. *In:* W. E. Enker (ed.), Carcinoma of the Colon and Rectum, pp. 21–48. Chicago: Year Book Medical Publishers, 1978.
18. Brodt, P. Characterization of two highly metastatic variants of the Lewis lung carcinoma with different organ specificities. Cancer Res., *46:* 2442–2448, 1986.
19. Brodt, P. The selection of a liver colonizing, highly metastatic subpopulation from the Lewis lung carcinoma variant H-59, using murine hepatocyte monolayers. Clin. Exp. Metastasis, *7:* 525–539, 1989.
20. Brodt, P. Tumor cell adhesion to frozen lymph node sections—an *in vitro* correlate of lymphatic metastasis. Clin. Exp. Metastasis, *7:* 343–352, 1989.
21. Marceau, N., Noel, M., and Deschenes, J. Growth and functional activities of neonatal and adult rat hepatocytes cultured in fibronectin coated substratum in serum-free medium. In Vitro, *18:* 1–11, 1982.
22. Shestowsky, W., Fallavollita, L., and Brodt, P. A monoclonal antibody to Lewis lung carcinoma variant H-59 identifies a plasma membrane protein with apparent relevance to lymph node adhesion and metastasis. Cancer Res., *50:* 1948–1953, 1990.
23. Albini, A., Iwamoto, Y., Kleinman, H. K., Martin, G. R., Aaronson, S. A., Kozlowski, J. M., and McEwan, R. N. A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. Cancer Res., *47:* 3239–3245, 1987.
24. Kimura, A. K., and Xiang, J. High levels of Met-72 antigen expression: correlation with metastatic activity of B-16 melanoma tumor cell variants. J. Natl. Cancer Inst., *76:* 1247–1253, 1986.
25. Harlow, E., and Lane, D. Monoclonal antibodies *In:* Antibodies—A Laboratory Manual, p. 139. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1988.
26. Monneron, A., and D'Alayer, J. Isolation of plasma and nuclear membranes of thymocytes. J. Cell Biol., *77:* 211–231, 1978.
27. King, J., and Laemmli, V. K. Polypeptides of the tail fibers of bacteriophage T4. J. Mol. Biol., 62: 465–477, 1971.
28. Towbin, H., Staehelin, T., and Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellular sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA, *76:* 4350–4354, 1979.
29. Tarentino, A. L., Gomez, C. M., and Plummer, T. H., Jr. Deglycosylation of asparginе-linked glycans by peptide: *N*-glycosidase F. Biochemistry, *24:* 4665–4671, 1985.
30. Springer, T. A., Dustin, M. L., Kishimoto, K., and Marlin S. D. The lymphocyte function-associated LFA-1, CD-2, and LFA-3 molecules: cell adhesion receptors of the immune system. Annu. Rev. Immunol., *5:* 223–252, 1987.
31. Roossien, F. F., de Rijk, D., Bikker, A., and Roos, E. Involvement of LFA-1 in lymphoma invasion and metastasis demonstrated with LFA-1-deficient mutants. J. Cell Biol., *108:* 1979–1985, 1989.
32. Schmirrmacher, V., Chiensong-Popov, R., and Arnheiter, H. Hepatocyte-tumor cell interaction *in vitro*. I. Conditions for rosette formation and inhibition by anti-H-2 antibody. J. Exp. Med., *151:* 984–989, 1980.
33. Roos, E., Van de Pavert, I. V., and Middelkoop, O. P. Infiltration of tumour cells into cultures of isolated hepatocytes. J. Cell Sci., *47:* 385–397, 1981.
34. Hashimoto, M., Niwa, O., Nitta, Y., Takeichi, M., and Yokoro, K. Unstable expression of E-cadherin adhesion molecules in metastatic ovarian tumor cells. Jpn. J. Cancer Res., *80:* 459–463, 1989.
35. Springer, R. A. Adhesion receptors of the immune system. Nature (Lond.), *346:* 425–434, 1990.
36. Newman, P. J., Berndt, M. C., II, Gorsky, J., White, G. C., Lyman, S., Paddock, C., and Muller, W. A. PECAM-1 (CD31): cloning and relation to adhesion molecules of the immunoglobulin gene superfamily. Science (Washington DC), *247:* 1219–1222, 1990.
37. Takeichi, M. The cadherins: cell-cell adhesion molecules controlling animal morphogenesis. Development (Camb.), *102:* 639–655, 1988.
38. Brandley, B. K., Swiedler, S. J., and Robbins, P. W. Carbohydrate ligands of the LEC cell adhesion molecules. Cell, *63:* 861–863, 1990.
39. Albelda, S. M., and Buck, C. A. Integrins and other cell adhesion molecules. FASEB, *4:* 2869–2880, 1990.
40. Roos, E., and Roossien, F. F. Involvement of leukocyte function-associated antigen-1 (LFA-1) in the invasion of hepatocyte cultures by lymphoma and T-cell hybridoma cells. J. Cell Biol., *105:* 553–559, 1987.
41. Kahn, H. J., Brodt, P., and Baumal, R. Lectin binding by liver and lung metastasizing variants of the murine Lewis lung carcinoma. Am. J. Pathol., *132:* 180–185, 1988.
42. Humphries, M. J., Matsumoto, K., White, S. L., and Olden, K. Oligosaccharide modification by swainsonine treatment inhibits pulmonary colonization by B16-F10 murine melanoma cells. Proc. Natl. Acad. Sci. USA, *83:* 1752–1756, 1986.
43. Dennis, J. W., Kosh, K., Bryce, D-M., and Breitman, M. L. Oncongenes conferring metastatic potential induce increased branching of Asn-linked oligosaccharides in rat 2 fibroblasts. Oncogene, *4:* 853–860, 1989.
44. Raz, A., Pazerini, G., and Carmi, P. Identification of the metastasis-associated, galactoside-binding lectin as a chimeric gene product with homology to an IgE-binding protein. Cancer Res., *49:* 3489–3493, 1989.
45. Kolb-Bachofen, V., Schlepper-Schafer, J., Roos, P., Hulsmann, D., and Kolb, H. GaLNAc/gal-specific rat liver lectins: their role in cellular recognition. Biol. Cell., *51:* 219–226, 1984.
46. Wang, J., and Brodt, P. Partial characterization by MoAb of tumor adhesion molecules which mediate attachment to hepatocytes *in vitro* and liver-colonization *in vivo*. Presented at the International Symposium on Critical Determinants in Cancer Progression and Metastasis (Abstract 15), Houston, TX, 1989.
47. McGuire, E. J., Mascali, J. J., Gardy, S. R., and Nicolson, G. L. Involvement of cell adhesion molecules in liver colonization by metastatic murine lymphoma/lymphosarcoma variants. Clin. Exp. Metastasis, *2:* 213–222, 1984.
48. Humphries, M. J., Yamada, K. M., and Olden, K. Investigation of the biological effects of anti-cell adhesive synthetic peptides that inhibit experimental metastasis of B16-F10 murine melanoma cells. J. Clin. Invest., *81:* 782–790, 1988.

# CHAPTER III:

## Inhibition of experimental hepatic metastasis by a monoclonal antibody that blocks tumor-hepatocyte interaction. *In vivo* event

Wang J, Fallavollita L, Brodt P.

Published in: Journal of Immunotherapy, 16:294-302, 1994

# Chapter III

## Summary:

To determine whether tumor cell adhesion to hepatocytes plays a role in H-59 metastasis to the liver *in vivo*, the effect of MAb C-11 on liver colonization was investigated. The results summarized in this chapter show that animals inoculated with MAb C-11 or its $F(ab)_2$ fragments had a significantly reduced number of hepatic metastases. This effect was antibody and organ specific. The inhibitory effect of MAb C-11 was also seen when it was inoculated directly into the animal by the i.v. or i.p. routes. This inhibitory effect resulted in an increase in the survival time of MAb C-11 treated animals. The effect of the antibody was not due to lymphocyte and / or monocyte-mediated ADCC or complement mediated lysis as determined by the use of F(ab)2 fragments and by *in vitro* analyses. Additional *in vitro* studies revealed that MAb C-11 could also block the stimulatory effect of hepatocytes on tumor cell proliferation seen in tumor-hepatocyte co-cultures. These result indicated that the adhesion of H-59 cells to hepatocytes through the plasma membrane glycoprotein receptor played a role in liver metastasis formation.

The *in vivo* experiments described in this chapter suggest that MAbs against tumor cell surface adhesion molecules could inhibit specific tumor-host cell interactions and could potentially provide an effective therapeutic tool in the treatment of disseminating cancer most likely in combination with other conventional therapy.

Journal of Immunotherapy
16:294–302 © 1994 Raven Press, Ltd., New York



# Inhibition of Experimental Hepatic Metastasis by a Monoclonal Antibody That Blocks Tumor–Hepatocyte Interaction

Jian Wang, Lucia Fallavollita, and *Pnina Brodt

*Department of Surgery, Division of Surgical Research and *the Division of Research, Department of Oncology, McGill University, Montreal, Quebec, Canada*

Summary: The role of tumor–hepatocyte interaction in carcinoma metastasis to the liver was investigated with use of the liver–metastatic murine carcinoma H-59 and a monoclonal antibody (MAb) C-11, which can inhibit tumor cell adhesion to hepatocytes in vitro by blocking a 64–71-kD glycoprotein receptor expressed on the tumor cell surface. The effect of this antibody on liver colonization by H-59 cells was analyzed. We found that treatment of H-59 cells with the antibody or with $F(ab)_2$ fragments prior to tumor cell inoculation markedly and specifically reduced the ability of the cells to form hepatic metastases. An inhibitory effect was also seen when the antibodies were administered directly to tumor-inoculated mice. In contrast, no reduction was seen in the number of lung metastases when the antibody-treated cells were inoculated intravenously. Studies in vitro revealed that coculture of the tumor cells with hepatocytes had a stimulatory effect on tumor cell proliferation that could be specifically blocked by MAb C-11. The results suggest that H-59 cell adhesion to hepatocytes via the plasma membrane receptor promotes liver metastases formation and provide further evidence that biological reagents that can abrogate specific tumor–host cell interactions may be beneficial in the prevention of tumor cell dissemination. Key Words: Metastasis—Adhesion—Monoclonal antibodies.

The potential of disseminating cancer cells to colonize a distant organ site is dependent on the outcome of multiple specific interactions between the tumor cells and different constituents of the target organ microenvironment including the microvascular endothelium (1,2), the subendothelial matrix (3), parenchymal cells (4), and paracrine growth factors (5,6). The liver is the primary site of metastasis for some of the most common human malignancies. Multiple liver metastases are often inoperable and respond poorly to chemotherapy resulting in poor prognosis (7). A better understanding of the cellular and molecular mechanisms underlying cancer dissemination to the liver may lead to the development of new, more specific and effective therapeutic strategies.

Tumor H-59—a subline of the Lewis lung carcinoma—metastasizes preferentially to the liver from local s.c. sites (8). Previously we have shown that liver metastasis formation in this model is associated with increased adhesion to hepatocytes, and a cell surface glycoprotein that mediates this attachment was subsequently identified by monoclonal antibody (MAb) C-11 (4,9). Since these studies were based mainly on in vitro analyses, we sought to confirm that tumor cell adhesion to hepatocytes

Received April 8, 1994; accepted October 6, 1994.

Address correspondence and reprint requests to Dr. P. Brodt at Department of Surgery, Division of Surgical Research, McGill University, 740 Dr. Penfield Ave., Montreal, Quebec H3A 1A4, Canada.

via this molecule was relevant to the metastatic process in vivo by testing the effect of the antibody on metastases formation. Our results show that MAb C-11 and its $F(ab)_2$ fragment blocked liver colonization. They suggest that tumor cell interaction with the hepatocytes promotes tumor cell growth in the liver and could provide a target for antibody-mediated intervention in the metastatic process.

## MATERIALS AND METHODS

### Mice

C57BL/6 female mice (7–12 weeks old) were used for all the experiments. They were purchased from Charles River Canada.

### Cell Lines

The origin and metastatic phenotype of tumor H-59 were described in detail previously (8). The tumor was maintained in vivo by s.c. implantation of liver metastases derived from tumor-bearing mice, into new recipient animals. Single-cell suspensions of the tumors were obtained by enzymatic digestion of the solid tumors in a 0.02% trypsin solution in $Ca^{2+}$ and $Mg^{2+}$ free phosphate-buffered saline (PBS) containing 0.2% EDTA, as we described previously (8). Where required, tumor cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS; Gibco), 0.01 *M* HEPES, $2 \times 10^{-3}$ *M* L-glutamine, and 0.002% gentamycin (RPMI-FCS). They were maintained in culture for up to 2 weeks prior to use in the experiments described.

### Antibodies

The production and antigenic specificities of MAb C-11, and MAb 12/50 [both mouse immunoglobulin (Ig)$G_1$] were described in detail in our previous papers (9,10). MAb 12/50 reacts with an $M_r$ 37,000 membrane protein of tumor H-59 but does not inhibit tumor cell adhesion to hepatocytes (10). MAb CL9002 (mouse $IgG_3$) to the H-$2D^b$ determinant expressed on tumor H-59 (9), MAb CL9007AP (mouse $IgG_{2b}$) to H-$2k^bD^b$, and the Low-Tox-R (rabbit) complement CL3061 were obtained from Cedarlane Laboratories Ltd. MAb J-2A2 (11), which was used as a control antibody in some of the experiments, was a kind gift from Dr. M. H. Hemler (The Dana Farber Cancer Institute). The dichlorotriazinylamino fluorescein (DTAF)-conjugated AffiniPure goat anti-mouse IgG (H + L) was obtained from Bio-Can Scientific. $F(ab)_2$ fragments of MAb C-11 were prepared as detailed previously (9). The Fab fragments used as controls were a kind gift from Dr. E. Roos (The Netherland Cancer Institute). They were prepared from a rabbit antiserum to mammary carcinoma TA3/Ha, which was reported to block the adhesion of TA3/Ha cells to hepatocytes and recognize integrin $\alpha_6\beta_4$ (12). In preliminary experiments, we found that they failed to block adhesion of H-59 cells to the hepatocytes.

### Tumor–Hepatocytes Adhesion Assay

Tumor cell adhesion to hepatocytes was measured as we previously described (9). To measure inhibition of adhesion by MAb C-11, the tumor cells were incubated with the antibody for 45 min on ice, washed three times to remove unbound antibody, and either added to the hepatocytes immediately or incubated first at 37°C for various time intervals as specified in the text. Adhesion was for 45 min at 37°C.

### Tumor Cells–Hepatocytes Coculture Assays

Hepatocytes were prepared and cultured in 24-well plates for 72 h as we previously described (4,9). The culture medium was removed and the hepatocytes washed repeatedly with, and then cultured for 24 h in, serum-free RPMI. To each well $2 \times 10^4$ H-59 cells grown in serum-depleted medium for 24 h were added, and the cells cocultured in RPMI medium for an additional 48 h with or without antibodies. The cells were pulsed for 18 h with 0.1 μCi [$^3$H]-thymidine (2 Ci/mmol, from Du Pont Canada) and lysed by repeated freezing and thawing, and the lysates were harvested onto paper filters with use of a Micromate 196 harvester (Packard). Thymidine incorporation was measured with use of a Beckman LS 8000 liquid scintillation counter. Background incorporation by hepatocytes was negligible. As controls, we used primary cultures of liver sinusoidal endothelial cells, prepared as described elsewhere (13), and confluent monolayers of the murine 3T6 fibroblasts (9), which were gamma-irradiated (2,500 R) 4 h prior to the addition of tumor cells.

### Experimental Hepatic Metastasis Assay

Tumor cells were inoculated by the intrasplenic (i.s.) route as means of delivering the cells into the

portal circulation, and the animals splenectomized immediately following injection, as originally described by Lafreniere and Rosenberg (14). When antibodies were used, the tumor cells were incubated with the ascites, with affinity-purified antibody or with $F(ab)_2$ fragments (as specified in the text) for 1 h at 4°C. Where indicated, the cells were washed four times prior to injection. Cell viability as assessed by the trypan blue exclusion dye exceeded 95%. In some experiments, animals were inoculated i.v. into the tail vein with MAb-treated cells. To test the effect of direct inoculation of antibodies, MAb C-11 was injected i.p. or i.v. prior to and following i.s. inoculation of tumor cells, as detailed in the text.

### Antibody-Dependent Cellular Cytotoxicity (ADCC) and Complement-Mediated Lysis Assays

$^{51}Cr$-labeled H-59 cells ($10^4$ cells/well) were added to 96-well V-bottomed microtiter plates (Libro/Titertek Microplates, Flow Laboratories). Syngeneic spleen cells were prepared and red blood cells removed with use of standard procedures (15). To each well, $10^6$ spleen cells or Low-Tox-R (rabbit) complement diluted 1:10 were added in a total volume of 200 μl RPMI containing 1% FCS, and the mixtures incubated for 4 h at 37°C. The plates were centrifuged for 5 min at 1,000 r/min, 50 μl of the supernatants removed from each well, and $^{51}Cr$ release monitored in a gamma counter.

Net cell lysis was calculated as follows: lysis (%) $= (a - b)/c \times 100$, where $a$ is cpm in test wells, $b$ is spontaneous release in control wells containing H-59 cells only, and $c$ is maximal release as measured by lysis of control H-59 cells with 0.1% SDS.

### Flow Cytometry

Tumor cells ($10^6$/ml) were incubated for 45 min on ice with 25 μg/ml of MAb C-11. Unbound antibody was removed by repeated washing and the cells either incubated immediately for 20 min on ice with DTAF-conjugated goat anti-mouse IgG (diluted 1:20) or maintained at 37°C (with occasional rocking) for the duration indicated in the text prior to addition of the second antibody. After three washes with PBS containing 0.1% bovine serum albumin (BSA) and 0.2% $NaN_3$, the cells were fixed with 1% paraformaldehyde and analyzed using a FACScan cell analyzer [Becton-Dickinson fluorescence-activated cell sorter (FACS) System] equipped with an argon-ion (15 MW) laser at 488 nm with a 530-nm filter. Data analysis was carried out with a Hewlett-Packard model 310 (9000 series) computer using the FACScan Research Program. Cells incubated with normal mouse IgG under identical conditions were used as negative controls. On the basis of measurements with H-59 cells labeled with the second antibody only, cells were defined as positive if their fluorescence intensity was >500 on a 0–1,023 channel scale.

### Statistical Analysis

The Mann–Whitney test was used for analysis of the metastasis data, the Wilcoxon Rank Sum test was used for survival data, and the Student $t$ test was used for analysis of the coculture assay.

## RESULTS

In testing the effect of MAb C-11 on the ability of H-59 cells to give rise to hepatic metastases, the tumor cells were treated with the antibody and inoculated by the i.s. route as means of delivering the cells into the portal vein (spleens were removed immediately following injection). The pooled results of six experiments, summarized in Fig. 1 show that animals inoculated with antibody-treated cells had a significantly reduced number of hepatic metastases as compared with animals injected with untreated cells or with cells pretreated with two other murine MAb ($p < 0.0002$). In experiments where a high concentration of antibody was used (150 μg/ml), 45% (10 of 22) of the mice injected with C-11–treated cells were tumor-free 13 days postinoculation when all mice in the control groups had multiple liver metastases (Fig. 1A).

To determine whether the effect of the antibody on liver metastases was mediated by Fc-dependent host immune mechanisms, we first tested the ability of MAb C-11 to lyse H-59 cells in the presence of complement or syngeneic spleen cells (ADCC). Results shown in Table 1 indicate that the antibody could not induce either complement or cell-mediated cytotoxicity. Furthermore, when $F(ab)_2$ fragments of the antibody that were previously shown to block tumor cell adhesion to hepatocytes in vitro (9) were used to treat H-59 cells prior to i.s. injection, we found that they were also effective in inhibiting liver colonization causing a significant reduction in the number of the metastases ($p < 0.01$).

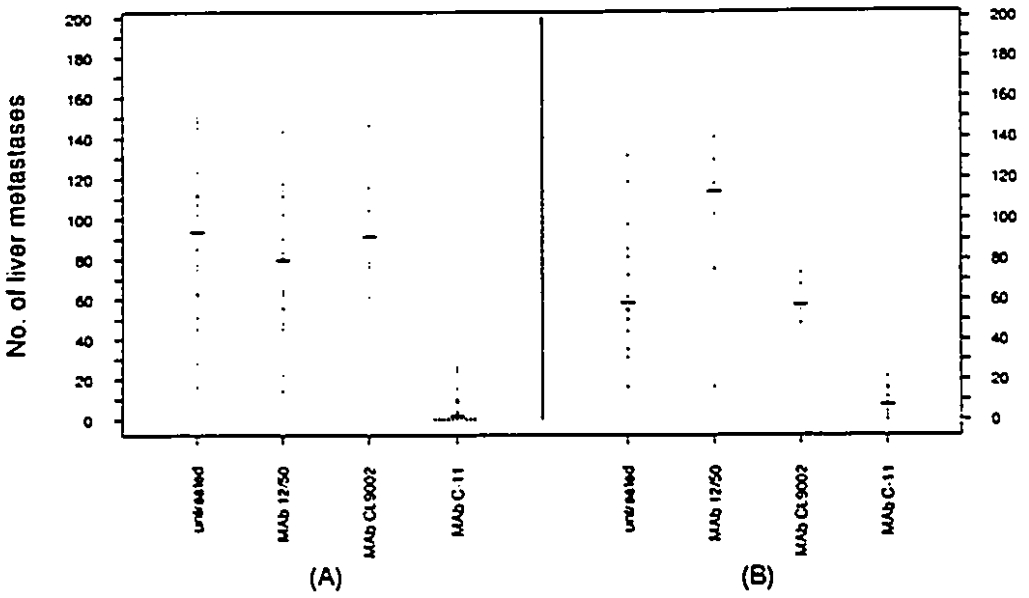



FIG. 1. Monoclonal antibody (MAb) C-11 inhibits experimental liver metastases formation by carcinoma H-59 cells. Animals were inoculated i.s. with $5 \times 10^4$ H-59 cells that were preincubated for 1 h at 4°C with a 1:20 dilution of ascites (A—total of three experiments) or 20–30 μg/ml affinity purified MAb (B–total of two experiments). Prior to injection cells were washed three times to remove unbound antibody. Splenectomy was performed 1 min following injection. Animals were sacrificed 13 days later and the metastases enumerated on the same day. The number of metastases in animals inoculated with MAb C-11–treated cells was significantly lower than in animals inoculated with untreated ($p < 0.0001$ for A and B), MAb 12/50 treated ($p < 0.0001$ for A and $<0.0004$ for B), or MAb CL9002 treated ($p < 0.0002$ for A and $<0.00012$ for B) cells. Each entry represents one animal. The horizontal bars denote medians.

Results of two experiments are shown in Fig. 2. In a third experiment, where animals were monitored until moribund, this inhibition resulted in an increase in the survival time of the treated animals (as shown in Fig. 3), although all the animals eventually died of residual liver metastases. In these experiments we also used Fab fragments of an antibody that had been shown to block the adhesion to hepatocytes of another tumor namely, rat carcinoma TA3/Ha (11). In vitro these fragments did not inhibit the adhesion of the H-59 cells to hepatocytes (results not shown). As shown in Figs. 2 and 3, they also had no effect on H-59 metastasis, suggesting that adhesion to hepatocytes and metastasis in the two systems were mediated by distinct receptors.

TABLE 1. *Monoclonal antibody (MAb) C-11 does not mediate antibody-dependent cellular cytotoxicity or complement dependent lysis of H-59 cells*

| H-59 Cells incubated with | % Lysis[a] |
|---|---|
| Spleen cells[b] | 3.7 ± 0.2% |
| Spleen cells + MAb C-11[c] | 2.1 ± 0.15% |
| Complement[c] | 2.9 ± 0.2% |
| Complement + MAb C-11 | 0.37 ± 0.2% |
| Complement + MAb CL9007AP[d] | 88 ± 0.63% |

[a] Results are means ± SD of triplicates.
[b] The ratio of target (H-59):effector (spleen) cells was 1:100.
[c] Antibodies were used at a concentration of 10 μg/ml and rabbit complement at a dilution of 1:10.
[d] MAb directed to H-2K$^b$D$^b$.

H-59 cells give rise to pulmonary metastases following the inoculation of high doses of cells i.v. into the tail vein (8). We tested whether pretreatment of the tumor cells with MAb C-11 could affect lung colonization. Results in Table 2 show that MAb C-11 had no effect on lung metastases formation. Under these experimental conditions, there was also no reduction in the number of liver metastases.

For further insight into the mechanism of MAb C-11–mediated inhibition of metastasis, it was of interest to know the duration of MAb retention on the tumor cell surface. Two in vitro assays were utilized. Flow cytometry was used to measure the proportion of labeled cells at different time intervals of up to 24 h following antibody binding, and an adhesion assay was used to monitor the biological activity of the antibody during the same time interval. As shown in Fig. 4, antibody was detectable on H-59 cells for up to 18 h following the initial labeling. However antibody-mediated inhibition of tumor cell adhesion began to decline at 3 h and was no longer evident 8 h after the initial MAb binding.

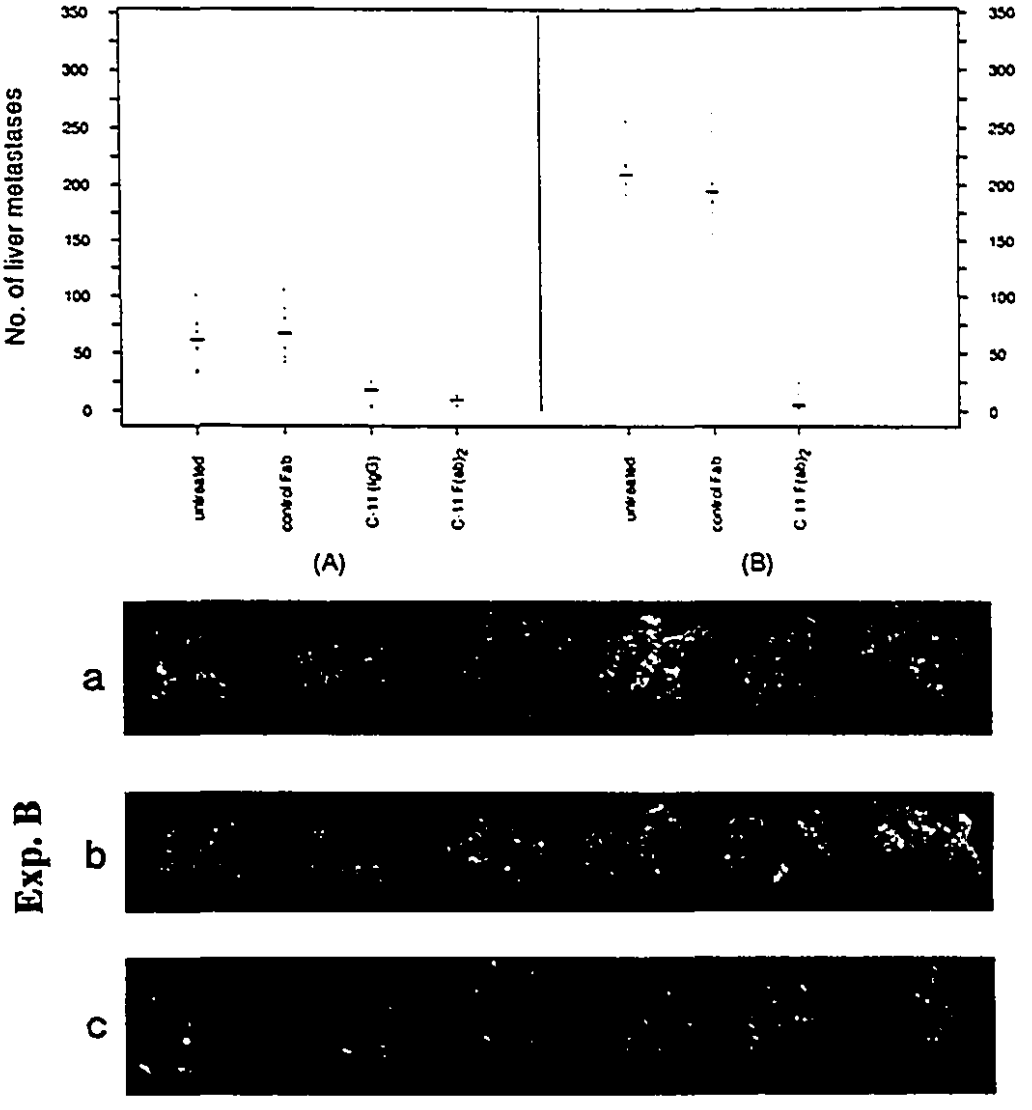



FIG. 2. $F(ab)_2$ fragments of monoclonal antibody (MAb) C-11 can block liver metastases formation. Top panel: One million H-59 cells were pretreated (as in the legend to Fig. 1) with 100 (Experiment A) and 200 μg/ml (Experiment B) MAb C-11 $F(ab)_2$ fragments (or control Fab). For C-11 IgG, a concentration of 50 μg/ml was used. The cells were readjusted to a concentration of $10^5$ cells/ml and the mice inoculated i.s. with 1 ml of the suspension each and splenectomized 1 min later. Mice were sacrificed 20 (A) and 13 (B) days after tumor inoculation and liver metastases enumerated on the same day. The difference in the number of metastases observed in the untreated groups of Experiments A and B is probably due to the longer duration of Experiment A, which resulted in the fusion of some smaller individual nodules. Bottom panel: The livers shown were from (a) untreated, (b) control Fab-treated, and (c) MAb C-11 $F(ab)_2$-treated mice in Experiment B.

For determining whether MAb C-11 could inhibit metastases formation when inoculated directly into the animals, it was administered i.p. or i.v. 24 and 4 h prior to, and 4 and 24 h following the i.s. injection of tumor cells. The rationale for the inoculation of antibodies prior to tumor cell injection was based on our previous finding that MAb C-11 also reacted with a hepatocyte cell surface determinant that was involved in the adhesion (9). The results shown in Table 3 demonstrate that treatment of the animals with MAb C-11 with use of either the i.v. or i.p. routes could significantly and specifically reduce ($p < 0.01$) the number of hepatic metastases.

To gain an understanding of the possible role that tumor cell adhesion to hepatocytes could play in the process of liver colonization, we tested in vitro the effect of coculture of tumor cells and hepatocytes on tumor cell proliferation. Serum-starved H-59

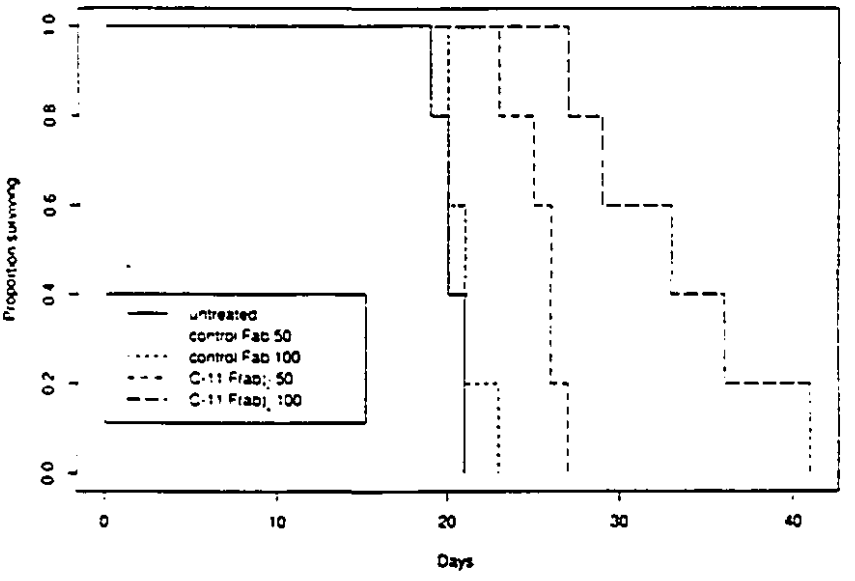



FIG. 3. Increased survival of mice inoculated with H-59 cells pretreated with $F(ab)_2$ fragments of monoclonal antibody (MAb) C-11. Animals (five per group) were inoculated i.s. with $10^5$ H-59 cells that were untreated or pretreated with 50 or 100 μg/ml of $F(ab)_2$ fragments. Mice inoculated with H-59 cells pretreated with either concentration of C-11 $F(ab)_2$ fragments but not with control Fab fragments survived significantly longer than those inoculated with untreated cells ($p = 0.01$).

cells were plated directly onto hepatocyte monolayers, and the cells cocultured for 48 h in the absence of a serum supplement. Results in Fig. 5 show that contact with the hepatocytes stimulated tumor cell proliferation increasing it 20-fold relative to control tumor cells cultured in the absence of hepatocytes, or 13-fold relative to cells cocultured with nonproliferating 3T6 fibroblasts or hepatic endothelial cells. The stimulatory effect of the hepatocytes was significantly reduced (56% reduction) in the presence of MAb C-11, whereas a MAb directed to the $H\text{-}2D^b$ determinant and an irrelevant murine ascites had no effect (Fig. 5). It is of interest that when cells of the Lewis lung carcinoma subline M-27, which are metastatic to lung but not to liver (8), were cocultured with hepatocytes the proliferative response observed was significantly lower ($p < 0.025$) (Fig. 5).

TABLE 2. *Monoclonal antibody (MAb) C-11 does not inhibit experimental lung metastasis of H-59 cells*

| | Metastases/lung | |
|---|---|---|
| Treatment of H-59 cells | Experiment 1 | Experiment 2 |
| None | 6 (0–23) | 157 (126–195) |
| MAb C-11[a] | 12 (0–21) | 146 (111–185) |

Animals were inoculated i.v. with $2 \times 10^5$ H-59 cells in Experiment 1 and with $5 \times 10^5$ cells in Experiment 2. They were sacrificed 21 days later. The results are expressed as the median (and range) of six animals in each group.

[a] There was no significant difference in the numbers of liver metastases seen in the two groups at the time of sacrifice. The number of liver metastases ranged from 0 to 2 in Experiment 1 and from 68 to 125 in Experiment 2.

## DISCUSSION

Several experimental tumor models have been used in recent years to demonstrate that the predilection of some metastatic cancer cells for the liver is associated with increased tumor cell adhesion to parenchymal and/or nonparenchymal liver cells (5,6,11). Much of this evidence is derived, however, from in vitro studies, and the relevance of these interactions to the metastatic process in vivo, particularly the importance of the tumor–hepatocyte interaction, has not been confirmed by experimental data.

In the present investigation, pretreatment of H-59 carcinoma cells with a MAb that specifically blocks tumor cell adhesion to hepatocytes in vitro (9) was used to determine whether this interaction plays a role in the metastatic process. We found that the antibody substantially reduced the number of liver metastases, suggesting that it either completely inhibited or significantly retarded tumor cell growth in the liver. This effect was antibody and organ specific, as demonstrated by the finding that treatment of H-59 cells with antibodies to two other plasma membrane proteins did not significantly alter the number of liver metastases (Fig. 1) and by the observation that MAb C-11 failed to block lung metastases formation (Table 1).

Lymphocyte- and monocyte-mediated ADCC and complement-mediated lysis were implicated in other studies where an inhibitory effect of antibodies in vivo was demonstrated (16). Our finding that $F(ab)_2$ fragments of the antibody also blocked the

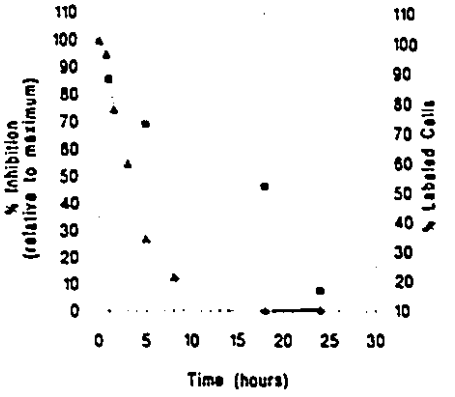



FIG. 4. A time-course analysis of cell surface-bound monoclonal antibody (MAb) C-11 as determined by flow cytometry and the loss of antibody function. H-59 cells were incubated for 45 min with 25 μg/ml purified MAb C-11 or an equivalent concentration of normal mouse immunoglobulin G (IgG) (for the adhesion assays) and anti H-2K$^b$D$^b$ MAb and then washed three times to remove unbound antibody. To measure adhesion (▲), the cells were either added directly to the hepatocytes or incubated at 37°C for the indicated time intervals. They were isotope labeled with $^{51}$Cr either before the addition of the antibody or (for 18- and 24-h intervals) before addition to hepatocytes. Adhesion assay was for 45 min at 37°C. At each time point, control untreated cells or cells treated with control IgG were also used and the inhibition calculated relative to adhesion levels seen with control untreated cells. Normal IgG and anti H-2K$^b$D$^b$ had no effect on adhesion at any time point and the basal level of adhesion of the untreated cells remained unchanged (30–40% of total) throughout the experiment. For fluorescence activated cell sorter (FACS) analysis (■), background labeling with mouse IgG was subtracted at each time point. During the first 5 h, mean fluorescence intensity of the labeled cells declined from 713 to 611.

growth of liver metastases suggests that MAb C-11 had a direct effect on the tumor cells that did not depend on Fc-mediated host immune mechanisms. This was also indicated by our findings that MAb C-11 failed to mediate lysis of H-59 cells in vitro in the presence of rabbit complement or normal syngeneic spleen cells (Table 1). The finding that all mice in the F(ab)$_2$ fragments–treated group eventually developed some liver metastases, whereas animals treated with whole IgG$_1$ molecules had a significantly reduced incidence (Fig. 1) may be related to a more rapid shedding of F(ab)$_2$ fragments from the cell surface (17) resulting in earlier re-expression of the receptors.

The observation that pretreatment of the tumor cells with the antibody prior to injection was sufficient to inhibit metastatic outgrowth suggests that the antibody interfered with an event occurring early in the process of liver colonization. This is also suggested by the lack of effect of the antibody on liver metastases formation when the cells were inoculated i.v. and could arrest in the lung microvasculature prior to recirculation into the liver through the arterial blood flow. Our previous studies have shown that MAb C-11 does not block adhesion of H-59 cells to either hepatic sinusoidal endothelial cells or to matrix proteins (9). In the present study, we found that whereas MAb C-11 was detectable on H-59 cells for up to 18 h following antibody binding, the ability of the antibody to block tumor cell adhesion to hepatocytes in vitro began to decline as early as 3 h and was lost by 8 h, suggesting that a critical concentration of antibody on the cell surface was required for complete inhibition of the adhesion. Together these findings imply that in vivo the interaction with hepatocytes that is disrupted by MAb C-11 may occur within hours of tumor cell arrest in the hepatic microvasculature, prior to cell division and/or the loss of the inhibitory antibody density from the cell surface. As the hepatic sinusoidal endothelium lacks a continuous basement membrane (18), this could conceivably be mediated by rapid invasion into the subendothelial space possibly involving the formation of pseudopodial extensions as has been shown in other models (19) and observed in our own in vitro studies (4). Previously we reported that H-59 cells constitutively express high levels of an $M_r$ 72,000 gelatinase and cathepsin L (20). These enzymes may contribute to the invasiveness and migration of the tumor cells during these early stages of liver colonization.

Although increased tumor cell adhesion to hepatocytes has been observed in several experimental

TABLE 3. *Inhibition of liver metastasis by treatment of animals with monoclonal antibody (MAb) C-11*

| Animals | Metastases/liver | | |
|---|---|---|---|
| | Exp. 1[a] | Exp. 2[a] | Exp. 3[b] |
| Nontreated[c] | 112 (84–124) | 61 (26–114) | 102 (72–141) |
| MAb 12/50 | 118 (95–140) | ND | ND |
| MAb CL9007AP | ND | ND | 96 (59–138) |
| MAb C-11 | 35 (15–84) | 22 (8–44) | 4 (1–10) |

Results are expressed as median (and range) of the numbers of liver nodules of seven (Exp. 1), ten (Exp. 2), and six (Exp. 3) animals: $p < 0.001$ for Exp. 1 and $p < 0.01$ for Exp. 2 and 3. ND, not done. Exp., experiment.

[a] Animals received a total of 4 i.p. injections each of 0.2 ml MAb C-11 ascites (Experiment 2) or 32 μg/ml affinity purified antibody (Experiment 1). Two injections were administered 4 and 24 h prior to and two 4 and 24 h following the i.s. injection of $5 \times 10^4$ H-59 cells.

[b] Animals received a total of 4 i.v. injection of 25 μg purified MAb C-11 and were inoculated with $10^5$ H-59 cells with use of the same schedule as described for i.p. injections.

[c] Animals were injected with saline.

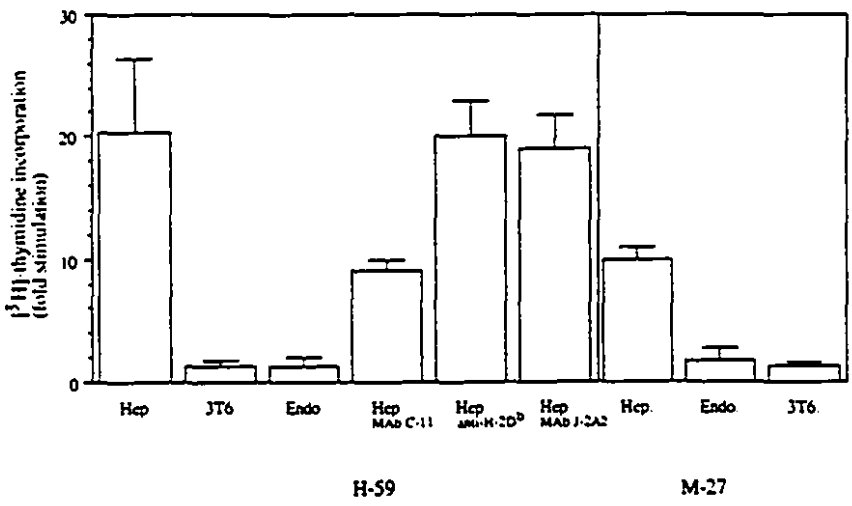



FIG. 5. Monoclonal antibody (MAb) C-11 blocks the proliferation of H-59 cells stimulated by coculture with hepatocytes. H-59 cells were cultured in serum-free RPMI medium for 24 h then dispersed and $2 \times 10^4$ cells added into wells of 24-well plates in which hepatocytes (Hep), 3T6 fibroblasts (3T6), or hepatic endothelial (Endo) cells were seeded 72 h earlier. The cells were cocultured for 48 h at 37°C and pulsed for 18 h with 0.1 μCi of [³H]-thymidine. Antibodies were added to the coculture after addition of the tumor cells, and the medium removed and replaced with fresh antibody solutions at 24 and 48 h. Final dilutions of the antibodies were 1:50 for ascites C-11 and J-2A2 and 1:100 for semipurified MAb CL9002. Endothelial cells and 3T6 monolayers were γ-irradiated (2,500 R) prior to addition of H-59 cells. Background [³H]-thymidine uptake by these cells and by the hepatocytes was negligible. When γ-irradiated hepatocytes were used as controls, no difference was seen in the level of stimulation of H-59 proliferation (not shown). [³H]-thymidine uptake by H-59 cells cocultured with hepatocytes was significantly higher ($p < 0.025$) than the uptake by M-27 cells cultured under the same conditions. It was significantly reduced ($p < 0.02$) by addition of MAb C11.

models of liver metastasis (4,6,11,21), the role that a tumor–hepatocyte interaction may play in the metastatic process is not yet known. Recently, we reported that hepatocyte-derived insulin-like growth factor I is highly mitogenic for H-59 cells (22). It is possible therefore that the hepatocytes regulate tumor cell growth in the liver through the release of growth factors. Tumor cell adhesion may in turn serve to maximize this paracrine stimulation by bringing the two cells into close proximity to each other. Further biochemical and molecular characterization of the receptor and ligand involved in the present tumor model, which are currently in progress, will provide a better understanding of the regulatory role that tumor cell adhesion to hepatocytes may play in liver metastasis.

Our finding that direct injection of antibodies into tumor-inoculated mice could also inhibit metastases formation suggests that the antibodies could react with receptor molecules and inhibit their function in vivo. Successful inhibition of metastases by antibodies directed to tumor adhesion receptors has recently also been reported by other laboratories (23,24). It is interesting to note in this context a recent study by De Potter et al. (25) showing that a monoclonal antibody raised to a cell-surface adhesion receptor expressed on the breast carcinoma cell line MCF-7 blocked tumor cell adhesion and invasion in vitro and specifically immunostained tissue biopsies derived from highly invasive ductal breast carcinomas. Staining was found to be particularly intense on long plasma membrane extensions protruding from the carcinoma cells, suggesting that the cell-surface determinant recognized by the antibody was involved in the invasive process in situ. Taken collectively these studies argue that antibodies that are raised against tumor cell surface adhesion molecules and can inhibit cellular adhesion and invasion mechanisms could potentially provide an effective therapeutic tool in the clinical management of cancer, probably in conjunction with conventional therapy.

Acknowledgment: The authors thank Dr. John Sampalis (Department of Surgery) and Dr. Sungsub Choi (Department of Epidemiology and Biostatistics) McGill University for their help with the analysis of the data, and Dr. J. Gordon (Department of Surgery, McGill University) for his help with the FACS analysis. This work was supported by a grant from the Medical Research Council of Canada. During the period in which this work was carried out P.B. was a Chercheur Boursier of the Fonds de la Recherche en Santé du Québec.

## REFERENCES

1. Haq M, Goltzman D, Tremblay G, Brodt P. Rat prostate adenocarcinoma cells disseminate to bone and adhere preferentially to bone marrow-derived endothelial cells. *Cancer Res* 1992;52:4613–9.
2. Zhu D, Pauli BU. Correlation between the lung distribution patterns of Lu-ECAM-1 and melanoma experimental metastases. *Int J Cancer* 1993;53:628–33.
3. Cerra RF, Nathanson SD. Organ-specific chemotactic factors present in lung extracellular matrix. *J Surg Res* 1989; 46:422–6.
4. Brodt P. Selection of a highly metastatic liver-colonizing subpopulation of Lewis lung carcinoma variant H-59 using

murine hepatocyte monolayers. *Clin Expl Metastasis* 1989; 7:525–39.
5. Zetter BR. The cellular basis of site-specific tumor metastasis. *N Engl J Med* 1990;322:605–12.
6. Rusciano D, Burger MM. Why do cancer cells metastasize into particular organs? *BioEssays* 1992;14:185–94.
7. Cohen AM, Shank B, Friedman MA. Colorectal cancer. In: DeVita VT, Hellman S, Rosenberg S, ed. *Cancer: principles and practice of oncology*. Philadelphia: J.B. Lippincott, 1989:907.
8. Brodt P. Characterization of two highly metastatic variants of the Lewis lung carcinoma with different organ specificities. *Cancer Res* 1986;46:2442–8.
9. Wang J, Fallavollita L, Brodt P. Identification of a M, 64,000 plasma membrane glycoprotein mediating tumor H-59 adhesion to hepatocytes. *Cancer Res* 1991;51:3578–84.
10. Shestowsky W, Fallavollita L, Brodt P. A monoclonal antibody to Lewis lung carcinoma variant H-59 identifies a plasma membrane protein with apparent relevance to lymph node adhesion and metastasis. *Cancer Res* 1990;50:1948–53.
11. Hemler ME, Strominger JL. Monoclonal antibody reacting with immunogenic mycoplasma proteins present in human hematopoietic cell lines. *J Immunol* 1982;129:2734–8.
12. Kemperman H, Wijnands Y, de Rijk D, Ross E. The integrin $\alpha_6\beta_4$ on TA3/Ha mammary carcinoma cells is involved in adhesion to hepatocytes. *Cancer Res* 1993;53:3611–7.
13. Morin O, Patry P, Lafleur L. Heterogeneity of endothelial cells of adult rat liver as resolved by sedimentation velocity and flow cytometry. *J Cell Physiol* 1984;119:327–34.
14. Lafreniere R, Rosenberg SA. A novel approach to the generation and identification of experimental hepatic metastases in a murine model. *J Natl Cancer Inst* 1986;76:309–15.
15. Brodt P, Gordon J. Anti-tumor immunity in B lymphocyte-deprived mice. II. *In Vitro* studies of cell-mediated cytotoxicity in normal and deprived mice. *Cell Immunol* 1981;65: 20–32.
16. Woodhouse CS, Morgan AC Jr. Murine monoclonal IgG3 antibodies to human colorectal tumor-associated antigens: production and characterization of antibodies active in both antibody-dependent cellular cytotoxicity and complement-mediated cytolysis. *Cancer Res* 1989;49:2766–72.
17. Fujimori K, Covell DG, Fletcher JE, Weinstein JN. Modeling analysis of the global and microscopic distribution of immunoglobulin G, $F(ab)_2$, and Fab in tumors. *Cancer Res* 1989;49:5656–63.
18. Martinez-Hernandez A. The hepatic extracellular matrix. I. Electron immunohistochemical studies in normal rat liver. *Lab Invest* 1984;51:57–74.
19. Chambers AF, Schmidt EE, MacDonald IC, Morris VL, Groom AC. Early steps in hematogenous metastasis of B16F1 melanoma cells in chick embryos studied by high-resolution intravital videomicroscopy. *J Natl Cancer Inst* 1992;84:797–803.
20. Brodt P, Reich R, Moroz LA, Chambers AF. Differences in the repertoires of basement membrane degrading enzymes in two carcinoma sublines with distinct patterns of site-selective metastasis. *Biochem Biophys Acta* 1992;1139:77–83.
21. Roos E, Rossien FF. Involvement of leukocyte function-associated antigen-1 (LFA-1) in the invasion of hepatocyte cultures by lymphoma and T-cell hybridoma cells. *J Cell Biol* 1987;105:553–9.
22. Long L, Nip J, Brodt P. Paracrine growth stimulation by hepatocyte-derived insulin-like growth factor-1: a regulatory mechanism for carcinoma cells metastatic to the liver. *Cancer Res* 1994;54:3732–7.
23. Zhu D, Cheng CF, Pauli BU. Blocking of lung endothelial cell adhesion molecule-1 (Lu-ECAM-1) inhibits murine melanoma lung metastasis. *J Clin Invest* 1992;89:1718–24.
24. Seiter S, Arch R, Reber S, et al. Prevention of tumor metastasis formation by anti-variant CD44. *J Exp Med* 1993; 177:443–55.
25. De Potter CR, Schelfhout AM, De Smet FH, et al. A monoclonal antibody directed against a human cell membrane antigen prevents cell substrate adhesion and tumor invasion. *Am J Pathol* 1994;144:95–103.

# CHAPTER IV:

## ERp72, its expression and possible role in H-59 liver colonization - additional unpublished findings

Chapter IV

## Abstract

Proteins of the endoplasmic reticulum have previously been detected on the surface of some normal cells. In previous studies we found that MAb C-11 recognized a 71 kDa glycoprotein (designated C-11 BP) on the surface of H-59 cells. An internal amino acid sequence analysis performed on a single protein band which was separated from a partially purified plasma membrane preparation of H-59 cells and migrated in the 71 kDa region, identified a molecules with sequence identity to the protein disulfide isomerase (PDI) - related protein ERp72. To determine whether ERp72 is detectable on the tumor cell surface and assess the possible role of ERp72 in adhesion of H-59 cells to hepatocytes, several experiments were carried out. Flow cytometry analysis showed that approximately 60% of H-59 cells labeled positively with an antibody to ERp72. Furthermore a polyclonal antibody to ERp72 blocked H-59 adhesion to hepatocytes by up to 62%. While these results suggested the ERp72 may play a role in H-59 adhesion, immunodepletion assays with anti ERp72 antibody demonstrated that the molecule recognized by MAb C-11 is distinct from the ERp72. These results imply that H-59 adhesion to hepatocytes may be mediated through the cooperative activity of several cell surface molecules including ERp72.

## Introduction

ERp72 is a endoplasmic reticulum (ER) protein of the PDI family and shares sequence homology with protein disulfide isomerase (PDI), also known as ERp59 (Mazzarella et al., 1990; Haugejorden et al., 1991). PDI catalyses disulfide bond formation and the ERp72 is thought to have a similar function (Freedman, 1989).

Expression of ER resident proteins on the cell surface has been reported regardless of whether or not they contain a retention signal. One line of evidence came from work by Yoshimori and colleagues (Yoshimoro et al., 1990). Using immunogold electron microscopy they found that PDI was localized not only in the ER of rat exocrine pancreatic cells but also in the plasma membrane (PM) and organelles along secretory pathways. There were no differences in the biochemical and immunochemical properties of transported or authentic ER resident PDI. Anti-KDEL antibodies reacted with PDI in both the PM and the ER of rat pancreatic cells. The results suggested that the ER protein PDI could be exported from the ER to the PM in rat exocrine pancreatic cells despite the presence of the KDEL retention signal sequence.

Mandel and his group provided additional evidence that PDI could be exposed at the PM. Using Chinese hamster ovary (CHO) cells, they have shown that the cell surface PDI had a disulfide reductive cleavage function on disulfide linked [$^{125}$I] iodotyramine / poly (D-lysine) conjugates when these cells non-specifically bound the conjugate (Mandel et al., 1993). This cleavage was inhibited

by a MAb to PDI and by bacitracin - an antibiotic known to inhibit both the reductive and the oxidative functions of PDI. The same reductive process by a membrane associated PDI plays a role in the penetration of membrane-bound human immunodeficiency virus (HIV) as was also found by the same group (Ryser et al., 1994). This HIV infection of human lymphoid cells was markedly inhibited by the membrane -impermeant sulfhydryl blocker 5,5'-dithiobis (2-nitrobenzoic acid), by bacitracin and by anti-PDI antibody.

In addition to PDI, expression of ERp72 has been reported on neutrophil PM. This expression was responsible for neutrophil priming in response to binding antibody (Weisbart, 1992). The antibody that primed neutrophils was shown to bind ERp72 in the neutrophil membrane by immunoprecipitating the same 72 kDa protein from neutrophils that was percipitated by a known antibody to ERp72. The gene for the protein associated with neutrophil priming was sequenced and identified as the ERp72. The investigators concluded that membrane associated ERp72 may play a role in the signal transduction pathway leading to priming of human neutrophils.

We found that MAb C-11 recognized a 71 kDa glycoprotein (C-11 BP) on the H-59 tumor cell surface. An internal amino acid sequence analysis performed on a corresponding protein band of partially purified PM preparations of H-59 cells identified a protein with sequence identity to the PDI-related protein ERp72. The results suggested that ERp72 may be expressed on the H-59 cell surface and

prompted us to investigate further the surface expression of ERp72 on H-59 cells and the possible role of ERp72 in adhesion of tumor H-59 cells to hepatocytes.

## Materials and Methods

Antibodies and others reagents:

The production and antigenic specificity of MAb C-11 were described in Chapter II. Normal rabbit serum (NRS) and affinity - purified normal mouse IgG were obtained from Cedarlane Laboratory Ltd. (Hornby, Ontario, Canada). Anti-ERp72(c), a rabbit antiserum against the 16 C-terminal amino acid fragment of murine ERp72 was a kind gift from Dr. M. Green (Saint Louis University Health Science Center, St. Louis, MO, USA). Normal mouse serum (NMS) was prepared from C57BL/6 female mice according to Harlow and Lane (Harlow and Lane, 1988). Protein A - Sepharose - CL 4B was obtained from Pharmacia Biotechnology (Uppsala, Sweden). Ponceau S concentrate was obtained from Sigma Diagnostics (St. Louis, MO, USA).

Sequencing analysis of partially purified H-59 plasma membrane:

H-59 plasma membrane were partially purified by gel filtration using G-200 sephadex. The collected fractions were subjected to electrophoresis followed by silver staining and the appropriate fractions (fractions number 70-130, 0.6 ml each, containing 71 kDa protein band) were pooled and concentrated using a

Centriprep-10 concentrator (Amicon, USA). The proteins were separated by a second round SDS-PAGE under reducing condition and transferred to a Immobilon PVDF membrane (Millipore corporation, Bedford, USA). The position of the C-11 BP was estimated by Western blot analysis with MAb C-11 and the quantity of the expected band was estimated by a BSA standard. The membrane was stained with a Ponceau S solution (0.2% Ponceau S in 1% acetic acid) and the band which migrated in the position indicated by Western blot analysis with MAb C-11 was cut and subjected to an internal amino acid sequence analysis performed using an ABI 477A Protein Sequencer with 120A Online PTH-AA Analyzer. The protein band was digested with trypsin or lysylendopeptidase and the digested peptides were separated by microbore HPLC (analyzed by Harvard Microchemistry Facility, Cambridge, USA).

Flow cytometry:

Tumor cells ($10^6$/ml) were pre-washed with PBS containing 0.1% bovine serum albumin and 0.2% $NaN_3$ (immunofluorescence (IF) buffer) and were incubated for 45 min. on ice with anti-ERp72(c) antibody diluted 1:100 in IF buffer. MAb C-11 ascites was used for comparison at a dilution of 1:20. NRS and NMS were used as a controls for non-specific antibody binding. Unbound proteins were removed by 3 washes with IF buffer and the cells were incubated immediately for 20 min. on ice with dichlorotriazinylamino fluorescein (DTAF)-conjugated goat anti-mouse or anti-rabbit IgG (1:20 dilution). Following 3 additional washes, the

cells were fixed with 1% paraformaldehyde and analyzed using a FACS (Becton-Dickinson FACS system, Mountainview, California, USA) equipped with an argon-ion (15MW) laser at 488 nm line with a 530 nm filter. Data analysis was carried out with a Hewlett-Packard model 310 (9000 series) computer using the FACScan Research Program. Dead cells were excluded from analysis by electronic gating.

Adhesion assays:

Hepatocytes were prepared 48 hours before the adhesion assay as previously described (Brodt, 1989b). To test for inhibition of the adhesion by antibodies the tumor cells were incubated with the antibodies for 45 min. on ice. Following 4 washes, the antibody-treated cells were added to the hepatocyte monolayer for the adhesion assay.

Immunodepletion assay:

Anti-ERp72(c) antiserum (2µl) was coupled to 200 µl protein A sepharose beads by incubation overnight at 4°C with slow rotation. The antibody coupled protein A beads were added to H-59 PM protein (250µg) after 6 repeated washes with PBS. The mixture was incubated overnight at 4°C and the protein A beads removed by centrifugation at 2,000 RPM for 10 min. The supernatant was collected for Western blot analysis or for additional depletion assays (3 depletions in total). For Western blotting, MAb C-11 ascites was used at a dilution of 1:20,

and the anti-ERp72(c) antiserum as well as NRS were used at a dilution of 1:800. Probing was overnight at 4°C. Alkaline phosphatase-conjugated goat anti-mouse Ig or goat anti-rabbit Ig at a dilution of 1:2000 were used as secondary antibodies.

## Results

MAb C-11 recognizes a 71 kDa protein on the tumor H-59 cell surface. Partially purified H-59 PM preparations were separated by SDS-PAGE and a 71 kDa band corresponding to a band detected by MAb C-11 in parallel gels was subjected to internal amino acid sequence analysis. The analysis identified a protein with sequence identity to the protein disulfide isomerase (PDI) - related protein ERp72 (Fig. 1, page 108).

To determine whether ERp72 was indeed expressed on tumor H-59 cells, flow cytometry was carried out using a rabbit anti murine ERp72(c) antiserum. Results in Figure 2 show that approximately 60% of H-59 cells stained positively with the anti-ERp72(c) antibody (Fig. 2-A, page 109). Non-immune NRS labeled fewer than 5% of the cells while MAb C-11 (positive control) labeled 98% of the cells (Fig. 2-B, page 109).

To determine whether ERp72 played a role in tumor cells adhesion to hepatocytes, H-59 cells were pretreated with the anti-ERp72(c) antiserum prior to the adhesion assay. Results shown in Figure 3 (page 110) demonstrate that the anti-ERp72(c) antibody blocked adhesion of H-59 cells to hepatocytes. The percentage of inhibition was 62% at a dilution of 1:10. Under similar conditions

non-immune NRS had no effect on the adhesion, while MAb C-11 ascites also inhibited the adhesion as shown previously (67% inhibition).

To determine whether the molecules detected with anti-ERp72 antiserum and with MAb C-11 were identical, immunodepletion assays were performed in combination with Western blot analyses. Three consecutive immunodepletion procedures with anti-ERp72(c) antibody were performed on PM preparations of H-59. Results shown in Figure 4 show that following these procedure there was a gradual, specific quantitative reduction in ERp72 proteins in the H-59 PM preparations as determined by Western blot analysis (Fig. 4, page 111). On the other hand the quantitative reduction in the protein detected by MAb C-11 had no change suggesting that the proteins detectable by the two antibodies were distinct.

## Discussion

ERp72 is a member of the protein disulfide isomerase family of proteins, which was originally located in endoplasmic reticulum of mammalian cells. It has been reported that PDI and ERp72 could be expressed on the surface of some cells (Mandel et al., 1993; Weisbart, 1992). Their expression on tumor cell surface has not been studied. Their possible function on the cell surface is still not totally understood.

An internal amino acid sequence analysis of a 71 kDa protein band isolated from partially purified H-59 PM preparations showed sequence identity to ERp72

(Fig. 1, page 108). This prompted us to examine the relationship between the adhesion molecule identified by MAb C-11 and the ERp72 protein.

We found that ERp72 could be detected by flow cytometry on approximately 60% of H-59 cells (Fig. 2-A, page 109). Furthermore, the anti-ERp72(c) antiserum blocked H-59 cells adhesion to hepatocytes by up to 62% suggesting that the protein was involved in the adhesion (Fig. 3, page 110). However, the ERp72 molecule was distinct from C-11 BP since immunodepletion assays with anti-ERp72(c) antibody while they showed a gradual reduction in the detectable ERp72 protein band following 3 cycles of depletion, but had no effect on the detectable levels of C-11 BP (Fig. 4, page 111). Moreover, under these conditions it became evident that the molecular weight of C-11 BP and ERp72 were different.

The possible function of ERp72 in tumor - hepatocyte adhesion is not clear. Since ERp72 belongs to the PDI family, it is thought that ERp72 has similar functions to PDI. The best-known function of PDI is to catalyze the oxidoreduction of disulfide bonds in the ER, which leads to the correct folding of newly synthesized proteins. However, PDI has been detected on the surface of some cells (Yoshimori et al., 1990; Mandel et al., 1993), and was found to be identical to several other proteins such as 3,3',5-triiodo-L-thyronine-binding protein ($T_3BP$) (Freedman, 1989) which was also exposed on the plasma membrane where it can exert reductive functions. Studies have shown that membrane-associated PDI expressed on CHO cells could possess a disulfide

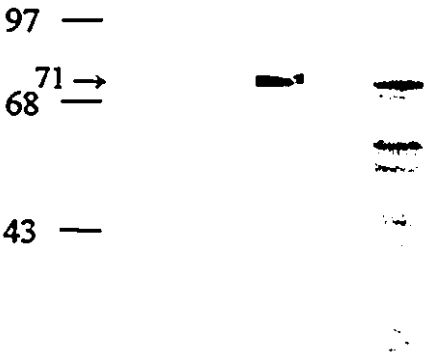
reductive cleavage function on a disulfide linked complex - [$^{125}$I] iodotyramine / poly (D-lysine) bound to the cells (Mandel et al., 1993). The cleavage was abolished by the membrane - impermeant sulfhydryl blocker 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB). This inhibitor can also prevent the cytotoxicity of diphtheria toxin (DT) -a disulfide - linked heterodimer which can bind to a specific surface receptor and must undergo chain separation in order to exert its cytotoxicity (Ryser et al., 1991). This suggests that a surface-associated reductive mechanism could activate DT by reducing the toxin's interchain disulfide bonds. The reductive process also plays a role in the penetration of membrane - bound human immunodeficiency virus (HIV) (Ryser et al., 1993). HIV infection of human lymphoid cells was markedly inhibited by anti-PDI antibodies and by DTNB. It is thought that HIV and its target cells engage in a thiol-disulfide interchange mediated by PDI and that the reduction of critical disulfides in viral envelope glycoproteins may be the initial event that triggers conformational changes required for HIV to fuse with the cell membrane and enter the cells.

The membrane - associated reductive cleavage function of ERp72 may also be of some functional relevance in our system since our data suggest that the C-11 BP on H-59 cells has intrachain disulfide bonds (Chapter II). It is also possible that on the H-59 cell surface, ERp72 may be located in close proximity to C-11 BP. ERp72 may play a disulfide reduction cleavage function to maintain C-11 BP in a structural conformation required for its adhesive function. This may explain the finding that adhesion was reduced when H-59 cell were pretreated with anti-

ERp72(c) antiserum as this treatment could block the function of ERp72 and affect indirectly the function of the C-11 BP. Alternatively it is conceivable that ERp72 plays no direct role in the adhesion and the inhibitory effect of anti ERp72 antibodies on adhesion was due to steric hindrance and some masking effect on the C-11 BP.

The role of ERp72 in adhesion may ultimately be clarified when the identity of the C-11 BP is determined. The data presented in this chapter are consistent with the conclusion that adhesion of H-59 cells to hepatocytes may be complex and involve multiple cell surface molecules.

(A).




(B).

| | 150 | 160 | 170 | 180 |
|---|---|---|---|---|
| **ERp72:** | -EEIVAKVREV | SQPDWTPPPE | VTLSLTK**DNF** | **DDVVNNADII** |
| **H-59:** | | | | **DNF DDVVNNADII** |

| | 190 | 200 |
|---|---|---|
| **ERp72:** | **LVEFYAPWCG** | HCKKLAPEYE- |
| **H-59:** | **LVEFYA** | |

**Figure 1: Amino acid internal sequencing of partially purified H-59 PM**

Partially purified H-59 PM preparations (containing 150 μg protein each) were separated by SDS-PAGE and transferred to a PVDF membrane. Internal amino acid sequence analysis was performed on a 71 kDa protein band (total 7 μ g). (A). Results of a Western blot analysis with MAb C-11. a: unfractionated H-59 PM. b: G-200 chromatographed H-59 PM. c: Ponseau S staining of fractionated H-59 PM. (B). Partial amino acid sequence of the 71 kDa protein and comparison to ERp72.

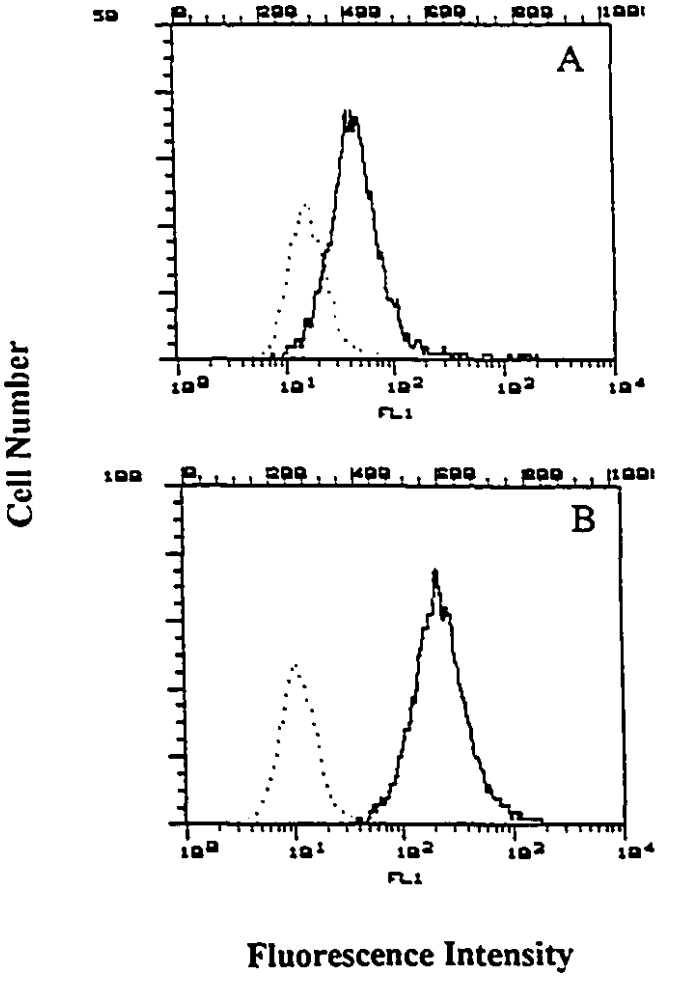



**Figure 2: Flow cytometric analysis of H-59 cells surface-bound anti-ERp72(c) antibody.**

H-59 cells were incubated with anti-ERp72(c) antiserum or NRS diluted 1:100 (A) or with MAb C-11 ascites and NMS diluted 1:10 (B). The dotted lines represent histograms obtained with cells incubated with normal serum while the solid line histograms show results obtained with test antibodies. Each profile is based on the analysis of 5000 cells and the abscissa shows the relative intensity of fluorescence. Approximately 60% of H-59 cells stained positively with anti-ERp72(c) antiserum while 98% of H-59 cell stained with MAb C-11 ascites. Only 5% and 2% of H-59 cells stained with NRS and NMS. The mean intensity of fluorescence values were about 60 for anti-ERp72 antiserum and about 200 for MAb C-11 ascites.

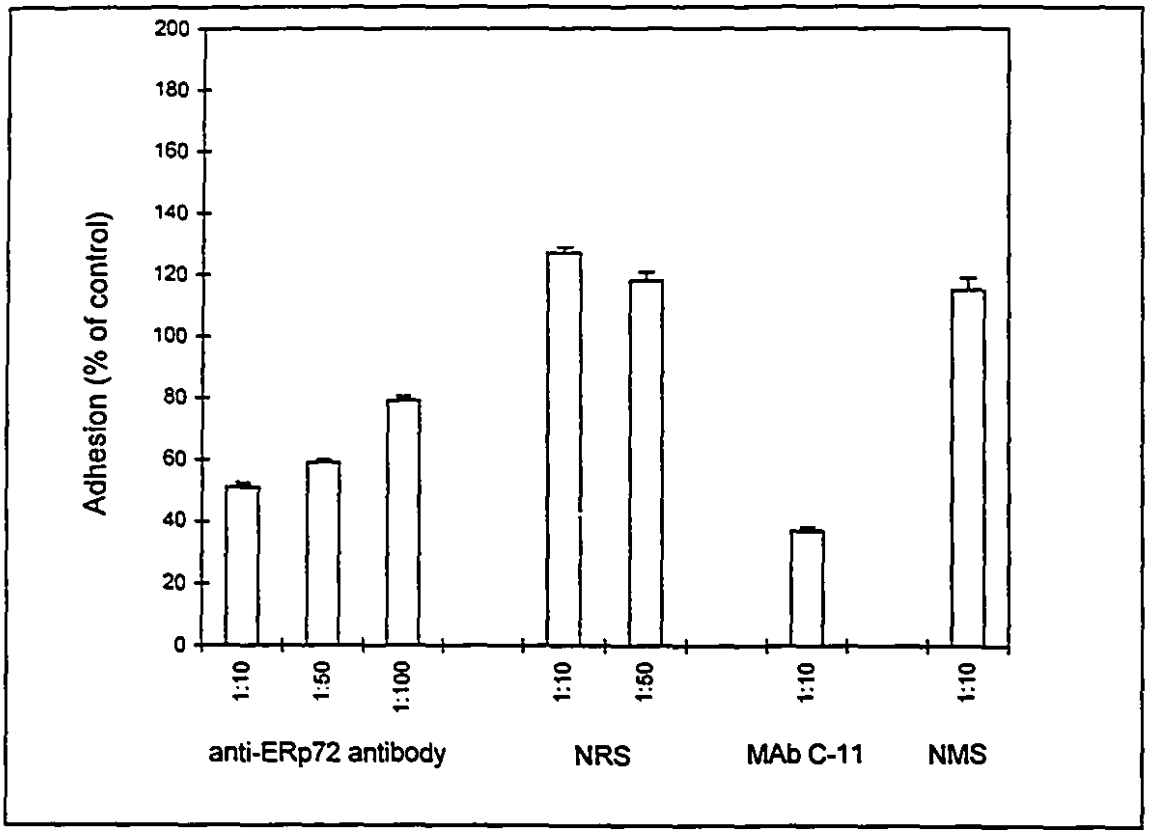



**Figure 3: The anti-ERp72 antibody blocks the adhesion of H-59 cells to hepatocytes**

H-59 cells were incubated with anti-ERp72(c) antiserum at dilutions of 1:10, 1:50 and 1:100. MAb C-11 ascites was diluted 1:10. The corresponding non-immune serum were used as controls. Results are expressed as percentage of adhesion obtained with non-treated H-59 cells and represent means and SD of triplicates.

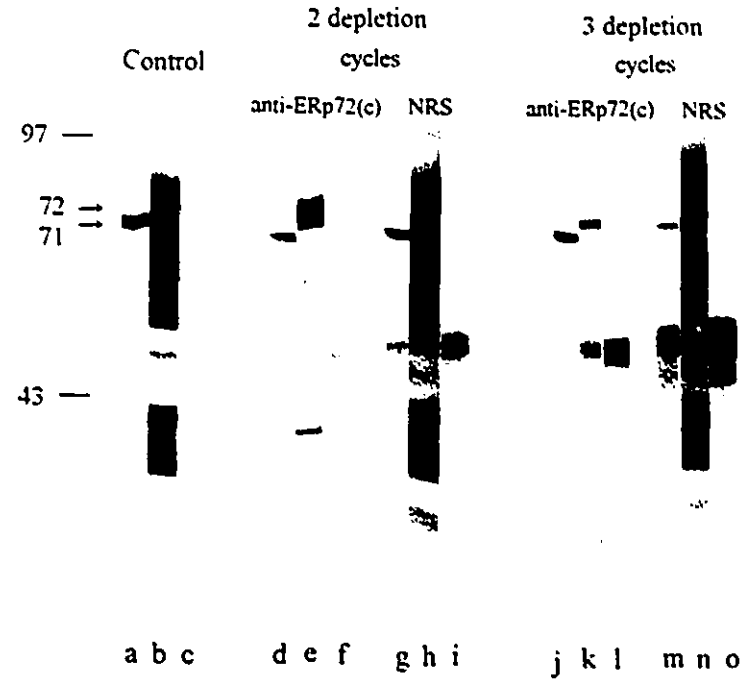



**Figure 4: ERp72 immunodepletion assays**

ERp72 proteins were depleted from H-59 PM preparations with rabbit anti-murine ERp72(c) antiserum using three cycles of immunoprecipitation. Depletion was monitered by Western blotting. Shown are: H-59 PM preparations before depletion (a-c), and after the second (d-i) and third (j-o) depletions. The SDS-PAGE separated and NC transferred proteins were probed with MAb C-11 ascites at a dilution of 1:20 (a, d, g, j, m), anti-ERp72(c) antibody (b, e, h, k, n) and NRS diluted 1:500 (c, f, i, l, o).

# CHAPTER V:

# Summary and Conclusions

# Chapter V

Cell adhesion molecules (CAM) mediating cell-cell and cell - extracellular - matrix interactions have been widely implicated in the process of malignant dissemination. Our study has focused on the role of cell-cell interactions between liver metastasizing carcinoma H-59 cells and the liver parenchymal cells i.e. the hepatocytes.

Using MAb C-11 produced by immunization with hepatocyte adherent H-59 cells (Fig. 5, page 74), we found that this MAb which could specifically block the adhesion of tumor cells to hepatocytes, recognized a 64-71 kDa plasma membrane glycoprotein (designated C-11 BP) with N-linked carbohydrate moieties constituting approximately 43% of its molecular mass.

The C-11 BP was also detected on hepatocytes and inhibition assays suggested that adhesion in this model required participation of the molecules expressed on both cell types. Therefore the adhesion between tumor cells and hepatocytes may be mediated by a homophilic recognition, or possibly by heterotypic adhesion involving receptors and counter receptors expressed on both cell types.

The cell adhesion molecules LFA-1 ($\alpha_L\beta_2$) (Meijne et al., 1994), $\alpha_5\beta_1$ (Kemperman et al., 1994) and $\alpha_6\beta_4$ (Kemperman et al., 1993) have been implicated in tumor cell - hepatocyte interactions in other tumor models. Our *in vitro* results indicated that C-11 BP is distinct from LFA-1 because MAb C-11 did

not react with lymphocytes and C-11 BP could not be detected in lysates of spleen cells and thymocytes. As well an anti-integrin $\beta_2$ MAb failed to block adhesion of H-59 cells to hepatocytes and an antibody to LFA-1 had a minimal inhibitory effect on adhesion of H-59 cells to hepatocytes. Integrin $\alpha_6\beta_4$ was also ruled out as a mediator of adhesion in our system since Fab fragments of an antiserum to $\alpha_6\beta_4$ which blocked adhesion to hepatocytes of another tumor cell line namely, mammary carcinoma TA3/Ha, failed to block adhesion of H-59 cells to hepatocytes and also did not inhibit H-59 metastasis to the liver. The molecular mass of the C-11 BP and the high content of N-linked oligosaccharides also suggest that the molecule is not an integrin.

Cell surface expression of N-linked oligosaccharides particularly the β,1-6 - branched N-linked oligosaccharides have been linked to the metastatic potential of tumor cells (Dennis et al., 1987). Our data revealed that although the N-linked carbohydrates in C-11 BP were not essential for MAb C-11 recognition they may have participated and played a role in the adhesion since enzymatic removal of cell surface carbohydrates could reduce adhesion of the tumor cells to hepatocytes. However, the possibility of involvement of other glycoconjugates expressed on the H-59 cell surface cannot be ruled out. A previous study showed that H-59 metastases had increased levels of the peanut agglutinin (PNA) binding carbohydrates β-D-galactose and N-acetyl-D-galactosamine as compared with the primary subcutaneous tumor, and the PNA binding molecules could also be found on hepatocytes (Kahn et al., 1988). It is possible that this hepatic lectin - binding

molecule which could be abnormally expressed on tumor H-59 cells is also involved in tumor-hepatocyte adhesion. Another liver lectin namely the asialoglycoprotein receptor has also been implicated in tumor cell adhesion to hepatocytes as well as to Kupffer cells and liver sinusoidal endothelial ceils (Kolb-Bachofen et al., 1984). It should be noted that treatment of H-59 cells with neuraminidase or β-galactosidase failed to modify adhesion, while treatment with both enzymes only reduced adhesion by 50% suggesting that the asialoglycoprotein receptor if it is involved plays only a partial role in the adhesion. The role of this receptor in H-59 adhesion remains to be investigated. Recently, the ganglioside GM2 has been detected on H-59 cells (Coulombe and Pelletier, 1993). Since this ganglioside is also a predominant ganglioside on normal mouse liver cells, it is conceivable that its expression on H-59 cells is functionally important for the establishment of liver metastasis.

Two cell surface molecules with a molecular mass similar to the C-11 BP which are also involved in cancer metastasis have been reported. One of these molecules is the 67 kDa laminin - binding protein isolated from the plasma membrane of different tumor cells and hepatocytes as well as from muscle cells (Tryggvason, 1993). Since MAb C-11 did not block H-59 cells binding to laminin (data not shown) or Matrigel (the major components of which are laminin and type IV collagen) coated culture dishes, it appears that the molecule recognized by C-11 is different from the laminin - binding protein. Another molecule with similarities to the C-11 BP is the 72 kDa oncofoetal glycoprotein 5T4 (Carsberg et

al., 1995). This antigen is defined by a MAb raised against human placental trophoblasts. It is expressed on many different carcinomas and in some normal epithelia and it contains about 39% N-linked carbohydrate moieties (Hole and Stern, 1990). However expression of this molecule could not be detected on liver tissue suggesting that it is probably not identical to the C-11 BP. Alternatively it is possible that the MAb used in the 5T4 study recognized an epitope not expressed on liver cells. Additional experiments such as blocking assays with anti-5T4 antibody in H-59 adhesion assays, antibody depletion analyses, and ultimately amino acid sequencing will determine the relationship between these two molecules.

The role of the tumor cell - hepatocyte interaction in liver metastasis was confirmed by our *in vivo* data. It was found that MAb C-11 which blocked this adhesion also inhibited liver metastases formation. This inhibitory effect was organ and antibody - specific.

MAbs have been reported to lyse tumor cells by Fc mediated ADCC or CMC host immune mechanisms (Goodman et al., 1990). This is not the case in our model since MAb C-11 did not lyse H-59 cells *in vitro* in the presence of complement or syngeneic spleen cells. Furthermore, the removal of the Fc portion of MAb C-11 did not affect the inhibitory function either *in vitro* or *in vivo*.

The molecular events which occur following arrest of tumor cells in the liver sinusoids and the role of tumor - hepatocyte adhesion are still a matter of speculation (Fig. 6, page 119). After they enter the sinusoidal vessels of the liver,

H-59 cells can adhere to sinusoidal endothelial cells through E-selectin - an inducible liver endothelial cell receptor (Brodt et al., 1994). H-59 cells can also produce ECM degrading enzymes, such as cathepsins B and L as well as gelatinase A to degrade ECM proteins (Brodt et al., 1992; Navab et al., 1995). These enzymes may contribute to the invasiveness and migration of the tumor cells and the establishment of cell-cell contact with the hepatocytes during the early stages of liver colonization. On the other hand, sinusoidal endothelial cells lack a continues BM and there is no BM around the hepatocytes (Sherlock and Dooley, 1993). Moreover, hepatocyte membrane microvilli can project through endothelial fenestrae for exposure to sinusoidal contents. Tumor cells can therefore interact directly with the hepatocytes through adhesion molecules such as the C-11 BP thereby facilitating liver metastases formation. In addition, hepatocytes can regulate tumor cell growth in the liver through the release of IGF-1 which can bind to the IGF-1 receptor expressed on H-59 cells (Long et al., 1994). Tumor cell adhesion to hepatocytes may enhance this paracrine stimulation by bringing the two cells into close proximity to each other. This is consistent with results shown by another group that tumor-hepatocytes contact was required for a growth stimulatory effect exerted by a diffusible hepatocyte factor on a liver-metastasizing subline of B-16 melanoma (Sargent et al., 1988).

While the C-11 BP remains to be fully characterized through amino acid sequencing or molecular cloning, the present results suggest that a second and possibly C-11 BP associated molecule namely ERp72 may also be involved in the

adhesion (Chapter IV). However the precise role of this molecule is unknown. The membrane - associated ER proteins PDI and ERp72 have been reported to be expressed on the surface of some cells (Weisbart, 1992; Mandel et al., 1993). Cell surface PDI has been shown to have a disulfide reductive cleavage function when PDI expressing cells bound to a disulfide - linked complex (Mandel et al., 1993). PDI may be involved in the activation of the toxic effect of membrane bound diphtheria toxin - a disulfide linked heterodimer, since the membrane - impermeant sulfhydryl blocker DTNB and anti-PDI antibody inhibited cytotoxicity of diphtheria toxin (Ryser et al., 1991; Mandel et al., 1993). PDI was also shown to be involved in HIV infection by reducing critical disulfide bonds in the viral envelope glycoproteins which may trigger conformational changes required for HIV to fuse with the target cell membrane (Ryser et al., 1993). Since C-11 BP contains intrachain disulfide bonds, it can speculated that ERp72 which may be located in close proximity to C-11 BP on the H-59 cell membrane plays some role maintaining the C-11 BP in a conformational state necessary for adhesion.

Other investigators have shown that antibodies directed to adhesion molecules on the tumor cell surface which can inhibit cell adhesion mechanisms such as tumor - endothelial cell or tumor cell - ECM interactions could also reduce experimental metastases formation (Haming et al., 1993; Newton et al., 1995). However, the present results provide the first indication that an antibody which blocks tumor - hepatocyte interactions can also be inhibitory *in vivo*. Our results demonstrated that MAb C-11 could inhibit liver metastases formation when the

tumor cells were pretreated before the intrasplenic injection or when injected directly by the i.v. or i.p. routes. Moreover, the inhibitory effect of the antibody resulted in an increase in survival of tumor - bearing animals. These results provide evidence that antibodies against tumor cell surface adhesion molecules which can inhibit cellular adhesion and invasion, could potentially provide an effective therapeutic tool in the clinical management of metastases probably in combination with conventional therapy.

Antibodies or other reagents directed to tumor cell adhesion molecules may have an additional advantage for therapy if they are specific for tumor cells or for particular secondary site. They may also be useful in the development of site-specific drug delivery systems as carriers can be designed to deliver hormones, drugs or toxins to particular sites using synthetic peptides or antibodies which recognize site - specific determinants. However, most cell adhesion molecules are also involved in normal physiological processes in inflammation, tissue repair and development where differential cell adhesion and migration are required. It is therefore important to test whether these new molecules also play a role in these physiological processes. Since the blocking effect of antibodies or other CAM inhibitors is likely to be temporary, high serum concentrations of these reagents need to be maintained through continuous infusion or by structure modification to increase their half life. Once these become possible, such anti-adhesion therapy directed at interfering with the adhesion of tumor cells to particular host cells may represent a major new approach to the prevention and / or treatment of metastases.

Future studies:

Final identification of the C-11 BP will only be possible once an amino acid sequence analysis is performed on the purified protein, a goal I was unable to achieve because of technical difficulties. This will require sufficient quantities of highly purified protein which can be obtained through immunoaffinity columns probably in combination with other fractionation procedures such as 2D gels or HPLC. Alternatively, H-59 λ gt11 cDNA expression library could be screened with MAb C-11 and the respective cDNA isolated and sequenced.

**Figure 6. Possible mechanisms involved in liver metastases formation by H-59 tumor cells**

After they enter the sinusoidal space of the liver, H-59 cells can adhere to sinusoidal endothelial cells through the interaction of E-selectin with its ligand. H-59 cells can also produce ECM degrading enzymes, such as cathepsins B and L as well as gelatinase A to degrade ECM proteins. This may facilitate H-59 cells adhesion to hepatocytes through the interaction of C-11 binding protein and its receptor. This adhesion may enhance growth stimulation by hepatocyte derived IGF-1. The membrane - associated ERp72 may also play some role in the adhesion. The expression of ganglioside GM2 by H-59 cells and adhesion of H-59 cells to laminin and fibronectin may also play a role in liver metastases formation. (Adapted from Sherlock and Dooley, 1993).

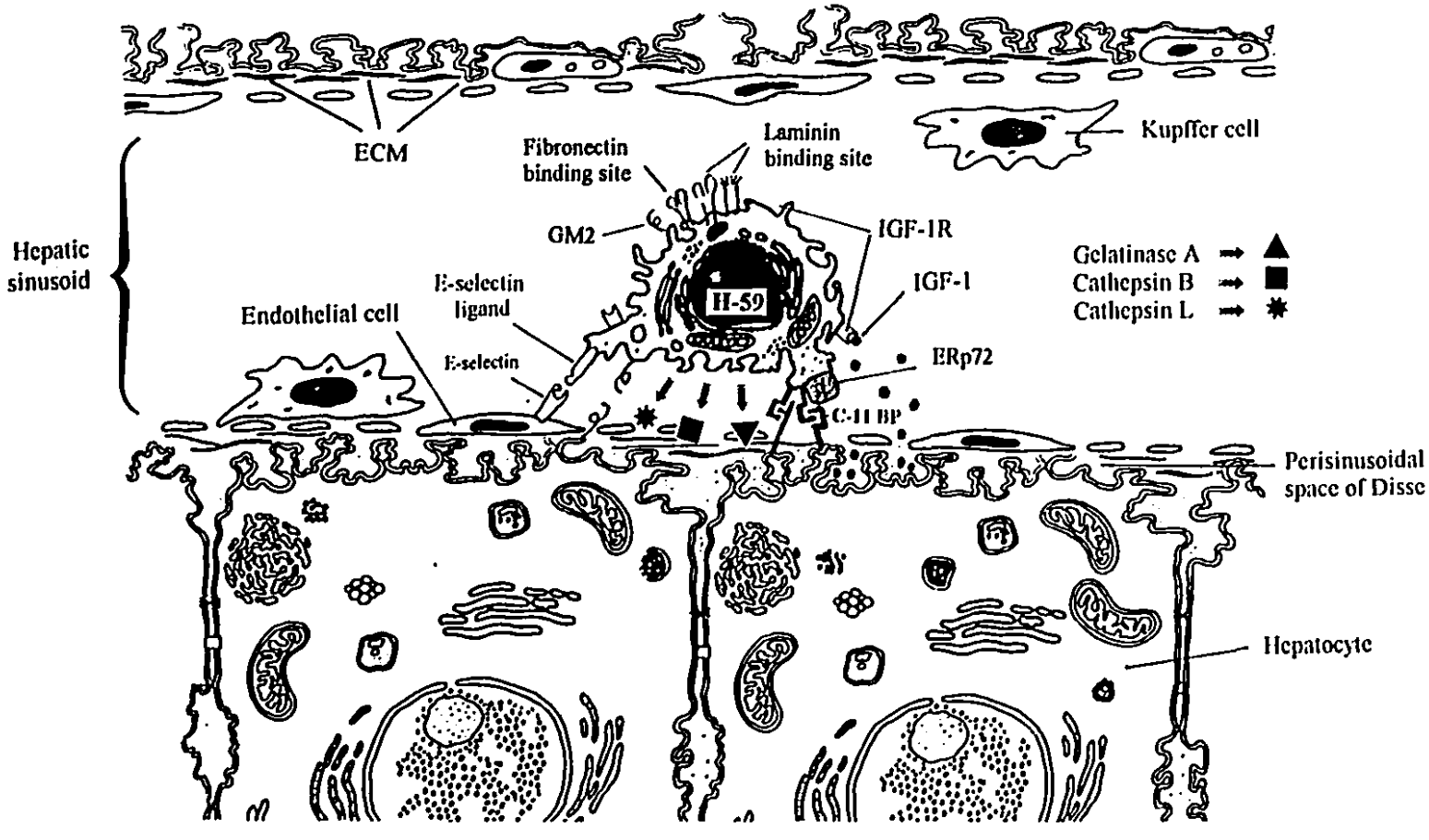



**Figure 6. Possible mechanisms involved in liver metastases formation by H-59 tumor cells**

# Bibliography

Aboud-Pirak E, Hurwitz E, Pirak M, et al: Efficacy of antibodies to epidermal growth factor receptor against KB carcinoma in vitro and in nude mice. **J Natl Cancer Inst** 1988; 80: 1605-1611.

Abrahamson DR, Caulfield JP: Distribution of laminin within rat and mouse renal, splenic, intestinal and hepatic basement membranes identified after the intravenous injection of heterologous anti laminin IgG. **Lab Invest** 1985; 52: 169-181.

Adams DH, Hubscher SG, Shaw J, et al: Intercellular adhesion molecule-1 on liver allografts during rejection. **Lancet** 1989; 2: 1122-1125.

Alsina AE, Bartus S, Hull D, et al: Liver transplant for metastatic neuroendocrine tumor. **J Clin Gastroenterol** 1990; 12: 533-537.

Aoki J, Umeda M, Takio K, et al: Neural cell adhesion molecule mediated contact-dependent inhibition of growth of near diploid mouse fibroblast cell line m5s/lM. **J Cell Biol** 1991; 115: 1751-1761.

Autio-Harmainen H, Karttunen T, Hurskainen T, et al: Expression of 72 kDa type IV collagenase (gelatinase A) in benign and malignant ovarian tumors. **Lab Invest** 1993; 63: 312-321.

Ballantyne GH, Quin J: Surgical treatment of liver metastases in patients with colorectal cancer. **Cancer** 1993; 71: 4252-4266.

Barberá-Guillem E, Alonso-Varona A, Vidal-Vanaclocha F: Selective implantation and growth in rats and mice of experimental liver metastasis in acinar zone one. **Cancer Res** 1989; 49: 4003-4010.

Barberá-Guillem E, Racha MA, Alvarez A, Vidal-Vanaclocha F: Differences in the lectin binding patterns of periportal and perivenous endothelial domains in the liver sinusoides. **Hepatology** 1991; 14: 131-139.

Barberá-Guillem E, Vidal-Vanaclocha F: Selective involvement of a specific sinusoidal domain in hepatic metastasis. II. Hepatic functional zones and their microvascular

implication. In: Orr FW, Buchanan MR, Weiss L (eds). Microcirculation in cancer metastasis. **CRC Press Inc.** 1991: 185-191.

Bartolazzi A, Cerboni C, Nicotra MR, et al: Transformation and tumor progression are frequently associated with expression of the $\alpha_3\beta_1$ heterodimer in solid tumors. **Int J Cancer** 1994; 58: 488-491.

Bates S, Davidson N, Valverius E, et al: Expression of TGF-$\alpha$ and its messenger RNA in human breast cancer: its regulation by estrogen and its possible functional significance. **Mol Endocrinol** 1988; 2: 543-555.

Behrens J, Mareel MM, Van Roy F, Birchmeier W: Dissecting tumor cell invasion: Epithelial cells acquire invasive properties after the loss of uvomorulin-mediated cell-cell adhesion. **J Cell Biol** 1989; 108: 2435-2447.

Behrens J: The role of cell adhesion molecules in cancer invasion and metastasis. **Breast Cancer Res Treat** 1993; 24: 175-184.

Benedetto A, Elia G, Sala A, Belardelli F: Hyposialylation of high-molecular-weight membrane glycoproteins parallels the loss of metastatic potential in wheat-germ agglutinin-resistant friend leukemia cells. **Int J Cancer** 1989; 43: 126-133.

Berchuck A, Kamel A, Whitaker R, et al: Overexpression of HER-2/new is associated with poor survival in advanced epithelial ovarian cancer. **Cancer Res** 1990; 50: 4087-4090.

Bevilacqua MP, Nelson RM: Endothelial leukocyte adhesion molecules in inflammation and metastasis. **Thromb Haemost** 1993; 70: 152-154.

Birch M, Mitchell S, Hart I: Isolation and characterization of human cell variants expressing high and low levels of CD44. **Cancer Res** 1991; 51: 6660-6667.

Birkedal-Hansen H, Moore WGI, Bodden MK, et al: Matrix metalloproteinases: a review. **Crit Rev Oral Biol Med** 1993; 4: 197-250.

Blumgart LH, Allison DJ: Resection and embolization in the management of secondary hepatic tumors. **World J Surg** 1982; 6: 32-45.

Brandley BK, Schnaar RL: Cell-surface carbohydrate in cell recognition and response. **J Leukoc Biol** 1986; 40: 97-111.

Brodt P: Characterization of two highly metastatic-variants of Lewis lung carcinoma with different organ specificities. **Cancer Res** 1986; 46: 2442-2448.

Brodt P: Tumor cell adhesion to frozen lymph node sections - an in vitro correlate of lymphatic metastasis. **Clin Exp Metastasis** 1989a; 7: 343-352.

Brodt P: Selection of a highly metastatic liver-colonizing subpopulation of Lewis lung carcinoma variant H-59 using murine hepatocyte monolayers. **Clin Exp Metastasis** 1989b; 7: 525-539.

Brodt P, Reich R, Moros LA, Chambers AF: Differences in the repertoires of basement membrane degrading enzymes in two carcinoma sublines with distinct patterns of site-selective metastasis. **Biochim Biophys Acta** 1992; 1139: 77-83.

Brodt P, Fallavollita L, Norton CR, Wolitzky BA: E-selectin mediates adhesion of carcinoma cells to the liver endothelium and liver metastasis (Abstract). **Proc Annu Meet Am Assoc Cancer Res** 1994; 35: 311.

Brodt P, Fallavollita L, Bresalier RS, et al: A monoclonal antibody to E-selectin blocks carcinoma cell adhesion to murine hepatic endothelial cells and inhibits experimental liver metastasis. Submitted.

Browder TM, Dunber CE, Nienhuis AW: Private and public autocrine loops in neoplastic cells. **Cancer Cells** 1989; 1: 9-17.

Brown PD, Levy AT, Margulies IMK, et al: Independent expression and cellular processing of Mr 72,000 type IV collagenase and interstitial collagenase in human tumorigenic cell lines. **Cancer Res** 1990; 50: 6184-6191.

Brown PD, Kleiner DE, Unsworth EJ, Stetler-Stevenson WG: Cellular activation of the 72 KDa type-IV procollagenase/TIMP-2 complex. **Kidney Int** 1993; 43: 163-170.

Carsberg CJ, Myers KA, Evans GS, et al: Metastasis-associated 5T4 oncofoetal antigen is concentrated at microvillus projections of the plasma membrane. **J Cell Sci** 1995; 108: 2905-2916.

Cavanaugh PG, Nicolson GL: Lung-derived growth factor that stimulates the growth of lung-metastasizing tumor cells: Identification as transferrin. **J Cell Biochem** 1991; 47: 261-271.

Chambers AF, Colella R, Denhardt DT, Wilson SM: Increased expression of cathepsins L and B and decreased activity of their inhibitors in metastatic, ras-transformed NIH 3T3 cells. **Mol Carcinog** 1992; 5: 238-245.

Chan BMC, Matsuura N, Takada Y, et al: In vitro and in vivo consequences of VLA-2 expression on Rhabdomyo-sarcoma cells. **Science** 1991; 251: 1600-1602.

Chang YS, Chen YQ, Timar J, et al: Increased expression $\alpha_{2b}\beta_3$ integrin in subpopulations of murine melanoma cells with high lung-colonizing ability. **Int J Cancer** 1992; 51: 445-451.

Charo IF, Nannizzi L, Smith JW, Cheresh DA: The vitronectin receptor $\alpha v\beta 3$ binds fibronectin and acts in concert with $\alpha_5\beta_1$ in promoting cellular attachment and spreading on fibronectin. **J Cell Biol** 1990; 111: 2795-2800.

Chaudhary V, Queen C, Junghans R, et al: A recombinant immunotoxin consisting of two antibcdy variable domains fused to Pseudomonas exotoxin. **Nature** 1989; 339: 394-397.

Chauhan SS, Goldstein LJ, Gottesman MM: Expression of cathepsin L in human tumors. **Cancer Res** 1991; 51: 1478-1481.

Cheresh DA, Klier FG: Disialoganglioside GD2 distributes preferentially into substrate-associated microprocesses on human melanoma cells during their attachment to fibronectin. **J Cell Biol** 1986; 102: 1887-1897.

Chung DC, Zetter BR, Brodt P: Lewis lung carcinoma variants with differing metastatic specificities adhere preferentially to different defined extracellular matrix molecules. **Invasion Metastasis** 1988; 8: 103-117.

Chuong CM, Edelman GM: Alterations in neural cell adhesion molecules during development of different regions of the nervous system. **J Neurosci** 1984; 4: 2354-2368.

Coulombe J, Pelletier G: Gangliosides and organ-specific metastatic colonization. **Int J Cancer** 1993; 53: 104-109.

Dejana E, Martin-Padura I, Lauri D, et al: Endothelial leukocyte adhesion molecule-1 dependent adhesion of colon carcinoma cells to vascular endothelium cells is

inhibited by an antibody to lewis fucosylated type-I carbohydrate chain. **Lab Invest** 1992; 66: 324-330.

Dennis JW: Effects of swainsonine and polyinosinic-polycytidylic acid on murine tumor cell growth and metastasis. **Cancer Res** 1986; 46: 5131-5136.

Dennis JW, Laferté S, Waghorne C, et al: β1-6 branching of Asn-linked oligosaccharides is directly associated with metastasis. **Science** 1987; 236: 582-585.

Dennis JW: Changes in glycosylation with malignant transformation and tumor progression. In: Fukuda M (ed). Cell surface carbohydrate and cell development. **CRC Press** 1991; pp. 161-194.

De Potter CR, Schelfhout AM, De Smet FH, et al: A monoclonal antibody directed against a human cell membrane antigen prevents cell substrate adhesion and tumor invasion. **Am J Pathol** 1994; 144: 95-103.

Dianzani U, Malavasi F: Lymphocyte adhesion to endothelium. **Crit Rev Immunol** 1995; 15: 167-200.

Dickson RB, Kasid A, Huff SE, et al: Activation of growth factor secretion in tumorigenic states of breast cancer induced by 17β-estradiol or v-Ha-ras oncogene. **Proc Natl Acad Sci USA** 1987; 84: 837-841.

Dillman RO: Chapter 13, Antibody therapy. In: Oldham RK (ed). Principles of cancer biotherapy. Second edition. **Marcel Dekker Inc.** 1991. pp. 395-432.

Divgi C, Wett S, Kris M, et al: Phase I and imaging trial of indium-111-labeled anti-epidermal growth factor receptor monoclonal antibody 225 in patients with squamous cell lung carcinoma. **J Natl Cancer Inst** 1991; 83: 97-104.

Drebin J, Link V, Greene M: Monoclonal antibodies specific for the new product directly mediate anti-tumor effects in vivo. **Oncogene** 1988; 2: 387-394.

East JA, Hart IR: CD44 and its role in tumor progression and metastasis. **Eur J Cancer** 1993; 29A: 1921-1922.

Edelman GM, Crossin KL: Cell adhesion molecules: Implications for a molecular histology. **Annu Rev Biochem** 1991; 60: 155-190.

Egan SE, Jarolim L, Rogelj S, et al: Growth factor modulation of metastatic lung colonization. **Anticancer Res** 1990; 10: 1341-1346.

Elices MJ, Osborn L, Takada Y, et al: VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. **Cell** 1990; 60: 577-584.

Emmert-Buck MR, Roth MJ, Zhuang Z, et al: Increased gelatinase A (MMP-2) and cathepsin B activity in invasive tumor regions of human colon samples. **Am J Pathol** 1994; 145: 1285-1290.

Fagnani R: The immunogenicity of foreign monoclonal antibodies in human disease applications: problems and current approaches. In: Goldfarb RH, Whiteside TL (eds). Tumor immunology and cancer therapy. **Marcel Dekker Inc.** 1994; pp. 3-22.

Feizi Ten: Demonstration by monoclonal antibodies that carbohydrate structures of glycoproteins and glycolipids are onco-developmental antigens. **Nature** 1985; 314: 53-57.

Fernandes B, Sagman U, Auger M, et al: β1-6 branched oligosaccharides as a marker of tumor progression in human breast and colon neoplasis. **Cancer Res** 1991; 51: 718-723.

Fitzer-Attas C, Feldman M, Eisenbach L: Expression of functionally intact PDGF-α receptors in highly metastatic 3LL Lewis lung carcinoma cells. **Int J Cancer** 1993; 53: 315-322.

Frankel AE, Houston LL, Issell BF: Prospects for immunotoxin therapy in cancer. **Annu Rev Med** 1986; 37: 125-142.

Freedman RB: Protein Disulfide Isomerase: Multiple roles in the modification of nascent secretory proteins. **Cell** 1989; 57: 1069-1072.

Frixen U, Behrens J, Sachs M, et al: E-cadherin mediated cell-cell adhesion prevents invasiveness of human carcinoma cell lines. **J Cell Biol** 1991; 111: 173-185.

Garbisa S, Pozzatti R, Muschel RJ, et al: Secretion of type IV collagenolytic protease and metastatic phenotype: induction by transfection with c-Ha-ras but not c-Ha-ras plus Ad2-Ela. **Cancer Res** 1987; 47: 1523-1528.

Gardner MJ, Jones LM, Catterall JB, Turner GA: Expression of cell adhesion molecules on ovarian tumor cell lines and mesothelial cells, in relation to ovarian cancer metastasis. **Cancer Lett** 1995; 91: 229-234.

Gasic GJ: Role of plasma, platelets, and endothelial cells in tumor metastasis. **Cancer Metastasis Rev** 1984; 3: 99-116.

Geerts A, Bouwens L, Wisse E: Ultrastructure and function of hepatic fat-storing and pit cells. **J Electron Microsc Tech** 1990; 14: 247-256.

Giancotti FG, Rouslahti E: Elevated levels of the $\alpha_5\beta_1$ fibronectin receptor suppress the transformed phenotype of Chinese hamster ovary cells. **Cell** 1990; 60: 849-859.

Giroldi LA, Mareel M, de Ruyter A, Schalken JA: E-cadherin transfection in prostatic tumor cells effect on invasiveness (Abstract). **Clin Exp Metastasis** 1992; 10 (Suppl 1): 42.

Goldenberg DM: Future role of radiolabeled monoclonal antibodies in oncological diagnosis and therapy. **Semin Nucl Med** 1989; 19: 3392-3396.

Goldenberg DM, Horowitz JA, Sharkey RM, et al: Targeting, Dosimetry, and radioimmunotherapy of B-cell lymphomas with iodine- $^{131}I$ labeled IL2 monoclonal antibody. **J Clin Oncol** 1991; 9: 548-564.

Goodman G, Hellström I, Brodzinsky L, et al: Phase I trail of murine monoclonal autibody L6 in breast, colon, ovarian, and lung cancer. **J Clin Oncol** 1990; 8: 1083-1092.

Gorelik E, Segal S, Feldman M: Growth of a local tumor exerts a specific inhibitory effect on progression of lung metastases. **Int J Cancer** 1978; 21: 617-625.

Groves RW, Allen MH, Ross EL, et al: Expression of selectin ligands by cutaneous squamous cell carcinoma. **Am J Pathol** 1993; 143: 1220-1225.

Guinec N, Dalet-Fumeron V, Pagano M: "In Vitro" study of basement membrane degradation by the cysteine proteinases, cathepsin B, B-like and L. Digestion of collagen IV, laminin, fibronectin, and release of gelatinase activities from basement membrane fibronectin. **Biol Chem Hoppe-Seyler** 1993; 374: 1135-1146.

Günthert U, Hofmann M, Rudy W, et al: A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. **Cell** 1991; 65:13-24.

Guo Y, Ma J, Wang J, et al: Inhibition of human melanoma growth and metastasis in vivo by anti-CD44 monoclonal antibody. **Cancer Res** 1994; 54: 1561-1565.

Hakomori SI: Tumor-associated antigens, their metabolism and organization. **Chem Phys Lipids** 1986; 42: 209-233.

Hale G, Dyer MJS, Clark MR: Remission in non-Hodgkin's lymphoma with reshaped human monoclonal antibody campath-1H. **Lancet** 1988; 2: 1394-1399.

Hamada J, Cavanaugh PG, Miki K, Nicolson GL: A paracrine migration-stimulating factor for metastatic tumor cells secreted by mouse hepatic sinusoidal endothelial cells: identification as complement component C3b. **Cancer Res** 1993; 53: 4418-4423.

Harlow E, Lane D: Chapter 6. Monoclonal Antibodies. In: Harlow E, Lane D (eds). A laboratory manual. Second edition. **Cold Spring Harbor Laboratory** 1988; pp. 172, 521-523.

Harning R, Myers C, Merluzzi VJ: Monoclonal antibodies to lymphocyte function-associated antigen-1 inhibit invasion of human lymphoma and metastasis of murine lymphoma. **Clin Exp Metastasis** 1993; 11: 337-342.

Haugejorden S, Srinivasan M, Green M: Analysis of the retention signals of two resident luminal endoplasmic reticulum proteins by in vitro mutagenesis. **J Biol Chem** 1991; 266: 6015-6018.

He C, Wilhelm SM, Pentland AP, et al: Tissue cooperation in a proteolytic cascade activating human interstitial collagenase. **Proc Natl Acad Sci USA** 1989; 86: 2632-2636.

Heicappell R, Podlinski J, Buszello H, Ackermann R: Cell surface expression and serum levels of intercellular adhesion molecule-1 in renal cell carcinoma. **Urol Res** 1994; 22: 9-15.

Hellström I, Brankovan V, Hellström KR: Strong antitumor activities of IgG3 antibodies to a human melanoma-associated ganglioside. **Proc Natl Acad Sci USA** 1985; 82: 1499-1502.

Hellström I, Beaumier PL, Hellström KE: Antitumor effects of L6, an IgG2a antibody that reacts with most human carcinomas. **Proc Natl Acad Sci USA** 1986; 83: 7059-7063.

Herlyn D, Herlyn M, Steplewski Z, et al: Monoclonal antibodies in cell-mediated cytotoxicity against human melanoma and colorectal carcinoma. **Eur J Immunol** 1979; 9: 657-659.

Herlyn D, Koprowski H: IgG2A monoclonal antibodies inhibit human tumor growth through interaction with effector cells. **Proc Natl Acad Sci USA** 1982; 79: 4761-4765.

Herlyn M, Kath R, Williams N, et al: Growth regulatory factors for normal, premalignant, and malignant human cells in vitro. **Adv Cancer Res** 1990; 54: 213-234.

Heuff G, van der Ende MB, Boutkan H, et al: Macrophage populations in different stages of induced hepatic metastases in rats: an immunohistochemical analysis. **Scand J Immunol** 1993; 38: 10-16.

Hill RP: 11. Metastasis. In: Tannock IF, Hill RP (eds). The basic science of oncology. **McGraw-Hill Inc. Health Professions Division** 1992; pp. 178-195.

Hole N, Stern PL: Isolation and characterization of 5T4, a tumor- associated antigen. **Int J Cancer** 1990; 45: 179-184.

Hong RL, Lee WJ, Shun CT, et al: Expression of CD44 and its clinical implication in diffuse-type and intestinal-type gastric adenocarcinomas. **Oncology** 1995; 52: 334-339.

Hostetter RB, Campbell DE, Chi K, et al: Carcinoembryonic antigen enhances metastatic potential of human colorectal carcinoma. **Arch Surg** 1990; 125: 300-304.

Houghton AN, Scheinberg DS: Monoclonal antibodies: Potential applications to the treatment of cancer. **Semin Oncol** 1986; 13: 165-179.

Houghton AN, Scheinberg DS: Chapter 63. Monoclonal antibodies. In: Moossa AR, Stephen C, Schimpff SC, Robson MC (eds). Comprehensive textbook of oncology Vol. 2, **Williams & Wilkins** 1991; pp. 601-606.

Howard EW, Bullen EC, Banda MJ: Preferential inhibition of 72 and 92 kDa gelatinase by tissue inhibitor of metalloproteinases-2. **J Biol Chem** 1991; 266: 13070-13075.

Hughes KS, Sugarbaker PH: 5. Resection of the liver for metastatic solid tumors. In. Rosenberg SA (ed). Surgical treatment of metastatic cancer. **J B Lippincott Company** 1987; pp. 125-164.

Hynes RO: The impact of molecular biology on models for cell adhesion. **BioEssays** 1994; 16: 663-669.

Igarashi S, Kawaguchi T: Ultrastructure of invasion of the adult rat hepatocyte monolayer by rat ascites hepatoma variant sublines. **Invasion Metastasis** 1993; 13: 132-146.

Inufusa H, Kojima N, Yasutomi M, Hakomori S: Human lung adenocarcinoma cell lines with different lung colonization potential (LCP) and a correlation between expression of sialosyl dimeric $Le^x$ (defined by Mab FH6) and LCP. **Clin Exp Metastasis** 1991; 9: 245-257.

Iwai K, Ishikura H, Kaji M, et al: Importance of E-selectin (ELAM-1) and sialyl $lewis^a$ in the adhesion of pancreatic carcinoma cells to activated endothelium. **Int J Cancer** 1993; 54: 972-977.

Izumi Y, Taniuchi Y, Tsuji T, et al: Characterization of human colon carcinoma variant cells selected for sialyl $Le^x$ carbohydrate antigen: liver colonization and adhesion to vascular endothelial cells. **Exp Cell Res** 1995; 216: 215-221.

Jatzko GR, Lisborg PH, Stettner HM, Klimpfinger MH: Hepatic resection for metastases from colorectal carcinoma-survival analysis. **Eur J Cancer** 1995; 31A: 41-46.

Jiang WG, Hallett MB, Puntis MCA: Hepatocyte growth factor/scatter factor, liver regeneration and cancer metastasis. **Br J Surg** 1993a; 80: 1368-1373.

Jiang WG, Lloyds D, Puntis MCA, et al: Regulation of spreading and growth of colon cancer cells by hepatocyte growth factor. **Clin Exp Metastasis** 1993b; 11: 235-242.

Jin L, Hemperly JJ, Lloyd RV: Expression of neural cell adhesion molecule in normal and neoplastic human neuroendocrine tissues. **Am J Pathol** 1991; 138: 961-969.

Jothy S, Yuan SY, Shirota K: Transcription of carcinoembryonic antigen in normal colon and colon carcinoma. In situ hybridization study and implication for a new in vivo functional model. **Am J Pathol** 1993; 143: 250-257.

Juneja HS, Schmalsteig FC, Lee S, Chen J: Vascular cell adhesion molecule-1 and VLA-4 are obligatory adhesion proteins in the heterotypic adherence between human leukemia / lymphoma cells and marrow stromal cells. **Exp Hematol** 1993; 21: 444-450.

Kahn HJ, Brodt P, Baumal R: Lectin binding by liver and lung metastasizing variants of the murine Lewis lung carcinoma. **Am J Pathol** 1988; 132: 180-185.

Kainz C, Kohlberger P, Sliutz G, et al: Splice variants of CD44 in human cervical cancer stage IB to IIB. **Gynecol Oncol** 1995; 57: 383-387.

Katagiri A, Watanabe R, Tomita Y: E-cadherin expression in renal cell cancer and its significance in metastasis and survival. **Br J Cancer** 1995; 71: 376-379.

Kataoka H, DeCastro R, Zucker S, et al: Tumor cell-derived collagenase-stimulatory factor increases expression of interstitial collagenase, stromelysin, and 72 kD gelatinase. **Cancer Res** 1993; 53: 3154-3158.

Kawakami H, Ito M, Miura Y, Hirano H: Expression of Lewis$^X$ sugar structure in the liver metastasis of mouse colon carcinoma (colon 26) cells. **Clin Exp Metastasis** 1994; 12: 129-133.

Kemeny N, Daly J, Reichman B, et al: Intrahepatic or systemic infusion of fluorodeoxy uridine in patients with liver metastases from colorectal carcinoma. **Ann Intern Med** 1987; 107: 459-465.

Kemler R: From cadherins to catenins: cytoplasmic protein interactions and regulation of cell adhesion. **Trans Genet** 1993; 9: 317-321.

Kemperman H, Wijnands Y, de Rijk D, Roos E: The integrin $\alpha_6\beta_4$ on TA3/Ha mammary carcinoma cells is involved in adhesion to hepatocyte. **Cancer Res** 1993; 53: 3611-3617.

Kemperman H, Wijanads Y, Meijne AM, Roos E: TA3/St, but not TA3/Ha, mammary carcinoma cell adhesion to hepatocyte is mediated by $\alpha_5\beta_1$, interacting with surface-associated fibronectin. **Cell Adhes Commun** 1994; 2: 45-48.

Kitamura K, Takahashi T, Miyagaki T, et al: Efficacy and specificity of a monoclonal antibody-drug conjugate in chemotherapy by intratumoral injection. **Jpn J Cancer Res** 1992; 83: 769-774.

Kitamura K, Miyagaki T, Yamaoka N, et al: The role of monoclonal antibody A7 as a drug modifier in cancer therapy. **Cancer Immunol Immunother** 1993; 36: 177-184.

Kobayashi H, Moniwa N, Sugimur M, et al: Effects of membrane-associated cathepsin B on the activation of receptor-bound prourokinase and subsequent invasion of reconstituted basement membranes. **Biochim Biophys Acta** 1993; 1178: 55-62.

Köhler G, Milstein C: Continuous cultures of fused cells secreting antibody of predefined specificity. **Nature** 1975; 256: 495-497.

Kojima N, Hakomori SI: Specific interaction between gangliotriaosylceramide (Gg3) and sialosyllactosylceramide (GM3) as a basis for specific cellular recognition between lymphoma and melanoma cells. **J Biol Chem** 1989; 264: 20159-20162.

Kojima N, Hakomori SI: Cell adhesion, spreading, and motility of GM-3 - expressing cells based on glycolipid-glycolipid interaction. **J Biol Chem** 1991; 266: 17552-17558.

Kolb-Bachofen V, Schlepper-Schafer J, Roos P, et al: GaLNAc/gal-specific rat liver lectins: their role in cellular recognition. **Biol Cell** 1984; 51: 219-226.

Kopper L, Hanh TV, Lapis K: Experimental model for liver metastasis formation using Lewis lung tumor. **J Cancer Res Clin Oncol** 1982; 103: 31-38.

Korczak B, Dennis JW: Inhibition of N-linked oligosaccharide processing in tumor cells is associated with enhanced tissue inhibitor of metalloproteinases (TIMP) gene expression. **Int J Cancer** 1993; 53: 634-639.

Kramer RH, Marks N: Identification of integrin collagen receptors on human melanoma cells. **J Biol Chem** 1989; 264: 4684-4688.

Kusukawa J, Sasaguri Y, Shima I, et al: Expression of matrix metalloproteinase-2 related to lymph node metastasis of oral squamous cell carcinoma. A clinicopathologic study. **Am J Clin Pathol** 1993; 99: 18-23.

Laferté S, Loh LC: Characterization of a family of structurally related glycoproteins expressing β1-6-branched asparagine-linked oligosaccharides in human colon carcinoma cells. **Biochem J** 1992; 283: 193-201.

Lauri D, Needham L, Martin-Padara I, Dejana E: Tumor cell adhesion to endothelial cells: Endothelial leukocyte adhesion molecule-1 as an inducible adhesion receptor specific for colon carcinoma cells. **J Natl Cancer Inst** 1991; 83: 1321-1324.

Lehninger AL: Chapter 11. Carbohydrates: Structure and biological function. In: Lehninger AL (ed). Principles of biochemistry. Third edition. **Worth Publishers Inc.** 1984; pp. 277-301.

Levy AT, Cioce V, Sobel ME, et al: Increased expression of the Mr. 72,000 type IV collagenase in human colonic adenocarcinoma. **Cancer Res** 1991; 51: 439-444.

Liotta LA: Tumor invasion and metastases: Role of the extracellular matrix. **Cancer Res** 1986; 46: 1-7.

Liotta LA: Introductory overview: The role of cellular proteases and their inhibition in invasion and metastasis. **Cancer Metastasis Rev** 1990; 9: 285-287.

Liotta LA: Cancer cell invasion and metastasis. **Sci Am** 1992; 266: 54-63.

LoBuglio A, Wheeler R, Trang J, et al: Mouse/human chimeric monoclonal antibodies in man: kinetics and immune response. **Proc Natl Acad Sci USA** 1989; 86: 4220-4224.

Long L, Nip J, Brodt P: Paracrine growth stimulation by hepatocyte-derived insulin-like growth factor-1: A regulatory mechanism for carcinoma cells metastatic to the liver. **Cancer Res** 1994; 54: 3732-3737.

Long L, Rubin R, Baserga R, Brodt P: Loss of the metastatic phenotype in murine carcinoma cells expressing an antisense RNA to the insulin-like growth receptor. **Cancer Res** 1995; 55: 1006-1009.

Lotz MM, Korzelius CA, Mercurio AM: Human colon carcinoma cells use multiple receptors to adhere to laminin: involvement of $\alpha_6\beta_4$ and $\alpha_2\beta_1$ integrins. **Cell Regul** 1990; 1: 249-257.

Majuri ML, Hakkarainen M, Paavonen T, Renkonen R: Carcinoembryonic antigen is expressed on endothelial cells. **APMIS** 1994; 102: 432-438.

Mandel R, Ryser HJP, Ghani F, et al: Inhibition of a reductive function of the plasma membrane by bacitracin and antibodies against protein desulfide-isomerase. **Proc Natl Acad Sci USA** 1993; 90: 4112-4116.

Marshall JF, Rutherford DC, McCartney ACE, et al: $\alpha_v\beta_1$ is a receptor for vitronectin and fibrinogen, and acts with $\alpha_5\beta_1$ to mediate spreading on fibronectin. **J Cell Sci** 1995; 108: 1227-1238.

Martinez-Hernandez A: The hepatic extracellular matrix. 1. Electron immunohistochemical studies in normal rat liver. **Lab Invest** 1984; 51: 57-74.

Mathie RT, Wheatley AM, Blumgart LH: Chapter 7. Liver blood flow: physiology, measurement and clinical relevance. In: Blumgart LH (ed). Surgery of the liver and biliary tract. Second edition. Vol. 1, **Churchill Livingstone** 1994; pp: 95-110.

Matsumoto Y, Saiki I, Makabe T, et al: Inhibitory effect of antimetastatic fusion polypeptide of human fibronectin on tumor cell adhesion to extracellular matrices. **Jpn J Cancer Res** 1991; 82: 1130-1138.

Matsushita Y, Hoff SD, Nudelman ED, et al: Metastatic behavior and cell surface properties of HT-29 human colon carcinoma variant cells selected for their differential expression of sialyl-dimeric $le^x$ antigen. **Clin Exp Metastasis** 1991; 9: 283-299.

Mazzarella RA, Srinivasan M, Haugejorden SM, Green M: ERp 72, an abundant luminal endoplasmic reticulum protein, contains three copies of the active site sequences of protein disulfide isomerase. **J Biol Chem** 1990; 265: 1094-1101.

McArdle CS, Hole D, Hansell D, et al: Prospective study of colorectal cancer in the west of Scoland: 10-year follow-up. **Br J Surg** 1990; 77: 280-282.

McCuskey PA, Kan Z, Wallace S: An electron microscopy study of Kupffer cells in livers of mice having Friend erythroleukemia hepatic metastases. **Clin Exp Metastasis** 1994; 12: 416-426.

Meijne AML, Driessens MHE, La Rivière G, et al: LFA-1 integrin redistribution during T-cell hybridoma invasion of hepatocyte cultures and manganese-induced adhesion to ICAM-1. **J Cell Sci** 1994; 107: 2557-2566.

Meyers WC, Jones RS: II. Neoplasms of the liver. In: Meyers WC, Jones RS (eds). Taxt book of liver and biliary surgery. **J B Lippincott Company** 1990; pp. 19-36, 93-130.

Miele ME, Bennett CF, Miller BE, Welch DR: Enhanced metastatic ability of TNF-alpha-treated malignant melanoma cells is reduced by intercellular adhesion molecule-1 (ICAM-1, CD54) antisense oligonucleotides. **Exp Cell Res** 1994; 214: 231-241.

Mignatti P, Rifkin DB: Biology and biochemistry of proteinases in tumor invasion. **Physiol Rev** 1993; 731: 161-195.

Mooy CM, Luyten GPM, Dejong PTVM, et al: Neural cell adhesion molecule distribution in primary and metastatic uveal melanoma. **Human Pathol** 1995; 26: 1185-1190.

Morris VL, Koop S, MacDonald IC, et al: Mammary carcinoma cell lines of high and low metastatic potential differ not in extravasation but in subsequent migration and growth. **Clin Exp Metastasis** 1994; 12: 357-367.

Morrison SL, Oi VT: Transfer and expression of immunoglobulin genes. **Annu Rev Immunol** 1984; 2: 239-256.

Mortarini P, Gismondi A, Santoni A, et al: Role of the $\alpha_5\beta_1$ integrin receptor in the proliferative response of quiescent human melanoma cells to fibronectin. **Cancer Res** 1992; 52: 4499-4506.

Natali P, Nicotra Mr, Cavaliere R, et al: Differential expression of intercellular adhesion molecule-1 in primary and metastatic melanoma lesions. **Cancer Res** 1990; 50: 1271-1278.

Navab R, Mort JS, Brodt P: Cysteine proteinases mediate tumor invasion in liver metastasis: Evidence from a murine carcinoma model (Abstract). **Proc Am Assoc Cancer Res** 1995, 601.

Neuberger MS, Rajewsky K: Activation of mouse complement by monoclonal mouse antibodies. **Eur J Immunol** 1981; 11: 1012-1016.

Newton SA, Reeves EJ, Gralnick H, et al: Inhibition of experimental metastasis of human breast carcinoma cells in athymic nude mice by anti-$\alpha_5\beta_1$, fibronectin receptor integrin antibodies. **Int J Oncology** 1995; 6: 1063-1070.

Nicolson GL: Cancer metastasis. Organ colonization and the cell-surface properties of malignant cells. **Biochim Biophys Acta** 1982; 695: 113-176.

Nicolson GL, Dulski KM: Organ specificity of metastatic tumor colonization is related to organ-selective growth properties of malignant cells. **Int J Cancer** 1986; 38: 289-294.

Nicolson GL: Cancer metastasis: Tumor cells and host organ properties important in metastasis to specific secondary site. **Biochem Biophys Acta** 1988a; 948: 175-224.

Nicolson GL: Organ specificity of tumor metastasis: Role of preferential adhesion, invasion and growth of malignant cells at specific secondary sites. **Cancer Metastasis Rev** 1988b; 7: 143-188.

Nicolson GL: Cancer progression and growth: relationship of paracrine and autocrine growth mechanisms to organ preference of metastasis. **Exp Cell Res** 1993; 204:171-180.

Nigam AK, Wrba F, Durbin H, et al: CEA is a modulator of adhesion in colorectal tumor cells (Abstract). **Gut** 1993; 34 (Suppl 4): S67.

Nip J, Shibata H, Loskutoff DJ, et al: Human melanoma cells derived from lymphatic metastases use integrin αvβ3 to adhere to lymph node vitronectin. **J Clin Invest** 1992; 90: 1406-1413.

Nip J, Rabbani SF, Shibata HR, Brodt P: coordinated expression of the vitronectin receptor and the urokinase-type plasminogen activator receptor in metastatic melanoma cells. **J Clin Invest** 1995; 95: 2096-2103.

Okahara H, Yagita H, Miyake K, Okumura K: Involvement of very late activation antigen 4 (VLA-4) and vascular cell adhesion molecule 1 (VCAM-1) in tumor necrosis factor alpha enhancement of experimental metastasis. **Cancer Res** 1994; 54: 3233-3236.

Olsnes S, Sandvig K: How protein toxins enter and kill cells. In: Frankel AE (ed). Immunotoxins. **Kluwer Academic** 1988; pp. 39-73.

Ozeki Y, Takishima K, Takagi K, et al: Immunohistochemical analysis of cathepsin B expression in human lung adenocarcinoma: the role in cancer progression. **Jpn J Cancer Res** 1993; 84: 972-975.

Paget S: The distribution of secondary growths in cancer of the breast. **Lancet** 1889; 1: 571-573.

Paku S, Lapis K: Morphological aspect of angiogenesis in experimental liver metastases. **Am J Pathol** 1993; 143: 926-936.

Perrotti D, Cimino L, Falcioni R, et al: Metastatic phenotype: growth factor dependence and Integrin expression. **Anticancer Res** 1990; 10: 1587-1598.

Phillips NC: Kupffer cells and liver metastasis. **Cancer Metastasis Rev** 1989; 8: 231-252.

Phillips ML, Nudelman E, Gaeta FCA, et al: ELAM-1 mediates cell adhesion by recognition of a carbohydrate ligand, sialyl-$Le^X$. **Science** 1990; 250: 1130-1132.

Pietersz GA, Krauer K, McKenzie FC: The use of monoclonal antibody immunoconjugates in cancer therapy. **Adv Exp Med Biol** 1994; 353: 169-179.

Pignatelli M, Durbin H, Bodmer WF: Carcinoembryonic antigen functions as an accessary adhesion molecule mediating colon epithelial cell-collagen interactions. **Proc Natl Asso Canc USA** 1990a; 87: 1541-1545.

Pignatelli M, Smith MEF, Bodmer WF: Low expression of collagen receptors in moderate and poorly differentiated colorectal adenocarcinomas. **Br J Cancer** 1990b; 61: 636-638.

Porte H, Chastre E, Prevot S, et al: Neoplastic progression of human colorectal cancer is associated with overexpression of the stromelysin-3 and BM-40/SPARC genes. **Int J Cancer** 1995; 64: 70-75.

Press OW, Eary JF, Badger CC, et al: Treatment of refractory non-Hodgkin's lymphomas with radiolabeled MB-1 (anti CD-37) antibody. **J Clin Oncol** 1989; 7: 1027-1038.

Qian F, Vaux DL, Weissman IL: Expression of the integrin $\alpha_4\beta_1$ on melanoma cells can inhibit the invasive stage of metastasis formation. **Cell** 1994; 77: 335-347.

Radinsky R: Paracrine growth regulation of human colon carcinoma organ-specific metastasis. **Cancer Metastasis Rev** 1993; 12: 345-361.

Ramadori G: The stellate cell (Ito-cell, fat storing cell, lipocyte, perisinusoidal cell ) of the liver. **Virchows Arch B Cell Pathol** 1991; 61: 147-158.

Ramming KP, O'Toole K: The use of implantable chemoinfusion pump in the treatment of hepatic metastases of colorectal cancer. **Arch Surg** 1986; 121: 1440-1444.

Rao LVM: Tissue factor as a tumor procoagulant. **Cancer Metastasis Rev** 1992; 11: 249-266.

Ravikumar TS, Kane R, Cady B, et al: Hepatic cryosurgery with intraoperative ultrasound monitoring for metastatic colon carcinoma. **Arch Surg** 1987; 122: 403-409.

Ravikumar TS, Steele G JR: Chapter 158: Liver metastases. In: Moossa AR, Stephen C, Schimpff SC, Robson MC (eds). Comprehensive textbook of oncology Vol. 2, **Williams & Wilkins** 1991; pp. 1625-1637.

Ray JM, Stetler-Stevenson WG: The role of matrix metalloproteases and their inhibitors in tumor invasion, metastasis and angiogenesis. **Eur Respir J** 1994; 7: 2062-2072.

Reichman L, Foote J, Winter G: Expression of an antibody Fv fragment in myeloma cells. **J Mol Biol** 1988; 203: 825-828.

Reynolds J, Del Vecchio S, Sakahara H, et al: Anti-murine antibody response to mouse monoclonal antibodies: Clinical findings and implications. **Nucl Med Biol** 1989; 16: 121-125.

Rodeck U, Herlyn M: Growth factors in melanoma. **Cancer Metastasis Rev** 1991; 10: 89-101.

Roitt I: Chapter 3. Molecules which recognize antigen. In: Roitt I (ed). Essential Immunology, Seventh edition. **Blackwell Scientific Publications** 1991; pp. 35-45.

Roossien FF, Bikker A, De Rijk D, et al: Involvement of LFA-1 in lymphoma invasion and metastasis demonstrated with LFA-1-deficient mutants. **J Cell Biol** 1989; 108: 1979-1985.

Rossi MC, Zetter BR: Selective stimulation of prostatic carcinoma cell proliferation by transferrin. **Proc Natl Acad Sci USA** 1992; 89: 6197-6201.

Rothlein R, Dustin ML, Marlin SD, Springer TA: A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1. **J Immunol** 1986; 137: 1270-1274.

Ruiz P, Dunon D, Sonnenberg A, Imhof BA: Suppression of mouse melanoma metastasis by EA-1, a monoclonal antibody specific for α6 integrins. **Cell Adhes Commun** 1993; 1: 67-81.

Ruoslahti E, Giancotti FG: Integrins and tumor cell dissemination. **Cancer Cells** 1989; 1: 119-126.

Rusciano D, Lorenzoni P, Burger MM: The role of both specific cellular adhesion and growth promotion in liver colonization by F9 embryonal carcinoma cells. **Int J Cancer** 1991; 48: 450-456.

Ryser HJP, Mandel R, Ghani F: Cell surface sulfhydryls are required for the cytotoxicity of diphtheria toxin but not of ricin in Chinese hamster ovary cells. **J Biol Chem** 1991; 266: 18439-18442.

Ryser HJP, Levy EM, Mandel R, DiSciullo GJ: Inhibition of human immunodeficiency virus infection by agents that interfere with thiol-disulfide interchange upon virus-receptor interaction. **Proc Natl Acad Sci USA** 1994; 91: 4559-4563.

Sargent NSE, Oestreicher M, Haidvogl H, et al: Growth regulation of cancer metastases by their host organ. **Proc Natl Acad Sci USA** 1988; 85: 7251-7255.

Sato H, Takino T, Okada Y, et al: A matrix metalloproteinase expressed on the surface of invasive tumor cells. **Nature** 1994; 370: 61-65.

Scaddan PB, Dufresne MJ: Characterization of cysteine proteases and their endogenous inhibitors in MCF-7 and adriamycin-resistant MCF-7 human breast cancer cells. **Invasion Metastasis** 1993; 13: 301-313.

Schachter H, Narasimhan S, Gleeson P, et al: Glycosyl transferases involved in the biosynthesis of protein-bound oligosaccharides of the asparagine-D glucosamine and serine (threonine)-N-acetyl-D-galactosamine types. In: Martonosi AN (ed). The enzymes of biological membranes. **Plenum Press** 1985: pp. 227-278.

Schipper JH, Frixen UH, Behrens J, et al: E-cadherin expression in squamous cell carcinomas of head and neck: inverse correlation with tumor differentiation and lymph node metastasis. **Cancer Res** 1991; 51: 6328-6337.

Schlepper-Schafer J, Springer GF: Carcinoma autoantigens T and Tn and their cleavage products interact with Gal/GalNAc-specific receptors on rat Kupffer cells and hepatocytes. **Biochem Biophys Acta** 1989; 1013: 266-272.

Schmitt M, Janicke F, Moniwa N, et al: Tumor associated urokinase-type plasminogen activator: biological and clinical significance. **Biol Chem Hoppe-Seyler** 1992; 373: 611-622.

Schwartz MA, Scheinberg DA, Houghton AN: Chapter 11: Monoclonal antibody therapy. In: Pinedo HM, Longo DL, Chabner BA (eds). Cancer chemotherapy and biological response modifiers. Annual 13, **Elsevier** 1992; 156-174.

Schwarz RE, Iwatsuki S, Herberman RB, Theresa L: Lymphokine-activated killer cell activity in patients with primary and metastatic malignant liver tumors. **Hepatology** 1989; 10: 221-227.

Seftor RE, Seftor EA, Gehlsen KR, et al: Role of the $\alpha_v\beta_3$ integrin in human melanoma cell invasion. **Proc Natl Acad Sci USA** 1992; 89: 1557-1561.

Seftor REB, Seftor EA, Stetler-Stevenson WG, et al: The 72 kDa type IV collagenase is modulated via differential expression of $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins during human melanoma cell invasion. **Cancer Res** 1993; 53: 3411-3415.

Segawa Y, Ohnoshi T, Ueoka H, Kimura I: Neural cell adhesion molecule expression and clinical features in small cell lung cancer: a semi-quantitative immunohistochemical approach using an immunogold-silver staining method. **Acta Med Okayama** 1993; 47: 281-287.

Seiter S, Arch R, Reber S, et al: Prevention of tumor metastasis formation by anti-variant CD44. **J Exp Med** 1993; 177: 443-455.

Sherlock S, Dooley J: Chapter 1: Anatomy and function. In: Sherlock S, Dooley J (eds). Diseases of the liver and biliary system. Ninth edition. **Blackwell Scientific Publications** 1993; pp. 1-16.

Sherman DM, Weichselbaum R, Order SE, et al: Palliation of hepatic metastases. **Cancer** 1978; 41: 2013-2017.

Shestowsky W, Fallavollita L, Brodt P: A monoclonal antibody to Lewis lung carcinoma variant H-59 identifies a plasma membrane protein with apparent relevance to lymph node adhesion and metastasis. **Cancer Res** 1990; 50: 1948-1953.

Shimizu Y, Van Seventer GA, Siraganian R, et al: Dual role of the CD44 molecule in T-cell adhesion and activation. **J Immunol** 1989; 143: 2457-2463.

Sires UI, Schmid TM, Fliszar CJ, et al: Complete degradation of type X collagen requires the combined action of interstitial collagenase and osteoclast-derived cathepsin B. **J Clin Invest** 1995; 95: 2089-2095.

Skerra A, Pluncknthun A: Assembly of a function immunoglobulin Fv fragment in *E. coli*. **Science** 1988; 240: 1038-1041.

Sloane BF, Rozhin J, Johnson K, et al: Cathepsin B: Association with plasma membrane in metastatic tumors. **Proc Natl Acad Sci USA** 1986; 83: 2483-2487.

Smets LA, Van Beek WP: Carbohydrates of the tumor cell surface. **Biochim Biophys Acta** 1984; 738: 237-249.

Spitler L, del Rio M, Khentigan A, et al: Therapy of patients with malignant melanoma using a monoclonal antimelanoma antibody-ricin A chain immunotoxin. **Cancer Res** 1987; 47: 1717-1723.

Springer TA, Dustin ML, Kishimoto TK, Marlin SD: The lymphocyte function-associated LFA-1, CD2 and LFA-3 molecules: Cell adhesion receptors of the immune system. **Annu Rev Immunol** 1987; 5: 223-252.

Springer TA: Adhesion receptors of the immune system. **Nature** 1990; 346: 425-434.

Steinbach F, Tanabe K, Alexander J, et al: The influence of cytokines on the adhesion of renal cancer cells to endothelium. **J Urol** 1996; 155: 743-748.

Stoolman LM: Adhesion molecules controlling lymphocyte migration. **Cell** 1989; 56: 907-910.

Sugarbaker EV: Patterns of metastasis in human malignancies. **Cancer Biol Rev** 1981; 2: 235-278.

Sugiura K, Stock CC: Studies in a tumor spectrum. III. The effect of phosphoramides on the growth of a variety of mouse and rat tumors. **Cancer Res** 1955; 15: 38-51.

Svennerholm L: Chromatographic separation of human brain gangliosides. **Neurochemistry** 1963; 10: 613-623.

Takeuchi K, Yamaguchi A, Urano T, et al: Expression of CD44 variant exons 8-10 in colorectal cancer and its relationship to metastasis. **Jpn J Cancer Res** 1995; 86: 292-297.

Tarentino AL, Gomez CM, Plummer TH: Deglycosylation of asparagine-linked glycans by peptide: N-Glycosidase F. **Biochemistry** 1985; 24: 4665-4671.

Tarin D, Matsumura Y: Recent advances in the study of tumor invasion and metastasis. **J Clin Pathol** 1994; 47: 385-390.

Tennenbaum T, Yuspa SH, Grover A, et al: Extracellular matrix receptors and mouse skin carcinogenesis: altered expression linked to appearance of early markers of tumor progression. **Cancer Res** 1992; 52: 2966-2976.

Tomita Y, Saito T, Saito K, et al: Possible significance of VLA-4 (alpha 4 beta 1) for hematogenous metastasis of renal-cell cancer. **Int J Cancer** 1995; 60: 753-758.

Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellular sheets: procedure and some applications. **Proc Natl Acad Sci USA** 1979; 76: 4350-4354.

Tranberg K, Bengmark S: Metastatic tumours of the liver. In: Blumgart LH (ed). Surgery of the liver and biliary tract. **Churchill Livingstone** 1994; Vol. 2; pp. 1385-1397.

Trauth BC, Klas C, Peters AMJ, et al: Monoclonal antibody-mediated tumor regression by induction of apoptosis. **Science** 1989; 245: 301-305.

Tressler RJ, Updyke TV, Yeatman T, Nicolson GL: Extracellular Annexin II is associated with divalent cation-dependent tumor cell-endothelial cell adhesion of metastatic RAW 117 large-cell lymphoma cells. **J Cell Biochem** 1993; 53: 265-276.

Tressler RJ, Yeatman T, Nicolson GL: Extracellular annexin VI expression is associated with divalent cation-dependent endothelial cell adhesion of metastatic RAW 117 large-cell lymphoma cells. **Expt Cell Res** 1994; 215: 395-400.

Tryggvason K: The laminin family. **Curr Opin Cell Biol** 1993; 5: 877-882.

Twining SS: Regulation of proteolytic activity in tissues. **Crit Rev Biochem Mol Biol** 1994; 29: 315-383.

Umbas R, Schalken JA, Aalders TW, et al: Expression of the cellular adhesion molecule E-cadherin is reduced or absent in high grade prostate cancer. **Cancer Res** 1992; 52: 5104-5109.

VanderElst I, Dennis JW: N-linked oligosaccharide processing and autocrine stimulation of tumor cell proliferation. **Exp Cell Res** 1991; 192: 612-621.

Van der Wurff AAM, Arends JW, Van der Linden EPM, et al: L-CAM expression in lymph node and liver metastases of colorectal carcinoma. **J Pathol** 1994; 172: 177-182.

Vollmers HP, Birchmeier W: Monoclonal antibodies inhibit the adhesion of mouse B16 melanoma cells in vitro and block lung metastasis in vivo. **Proc Natl Acad Sci USA** 1983a; 80: 3729-3733.

Vollmers HP, Birchmeier W: Monoclonal antibodies that prevent adhesion of B16 melanoma cells and reduce metastases in mice: crossreaction with human tumor cells. **Proc Natl Acad Sci USA** 1983b; 80: 6863-6867.

Vollmers HP, Imhof BA, Braun S, et al: Monoclonal antibodies which prevent experimental lung metastasis. Interference with the adhesion of tumor cells to laminin. **FEBS Lett** 1984; 172: 17-20.

Vriesendorp HM, Herpst JM, Germack MA, et al: Phase I/II studies of yttrium-labeled antiferritin treatment for end-stage Hodgkin's diseases, including Radiation Therapy Oncology Group 87-01. **J Clin Oncol** 1991; 9: 918-928.

Waldmann TA: Monoclonal antibodies in diagnosis and therapy. **Science** 1991; 252: 1657-1662.

Walz G, Aruffo A, Kolanus W, et al: Recognition by ELAM-1 of the sialyl-$Le^x$ determinant on myeloid and tumor cells. **Science** 1990; 250: 1132-1135.

Wawrzynczak EJ, Thorpe PE: Chapter 18: Monoclonal antibodies and therapy. In: Franks LM, Teich NM (eds) Introduction to the cellular and molecular biology of cancer. Second edition. **Oxford Medical Publication** 1991; pp. 471-509.

Weber E, Gunther D, Laube F, et al: Hybridoma cells producing antibodies to cathepsin L have greatly reduced potential for tumor growth. **J Cancer Res Clin Oncol** 1994; 120: 564-567.

Weber GF, Ashkar S, Glimcher MJ, et al: Receptor-ligand interaction between CD44 and osteopontin (Eta-1). **Science** 1996; 271: 509-512.

Weisbart RH: An antibody that binds a neutrophil membrane protein, ERp72, primes human neutrophils for enhanced oxidative metabolism in response to formyl-methionyl-leucyl-phenylalanin. Implications for ERp72 in the signal transduction pathway for neutrophil priming. **J Immunol** 1992; 148: 3958-3963.

Wendler F, Ozanne B: Human squamous cell lung cancers express increased epidermal growth factor receptors. **J Clin Invest** 1984; 74: 647-651.

Wewer UM, Albrechtsen R: Carcinoma-associated perisinusoidal Laminin may signal tumor cell metastasis to the liver. **Virchows Arch A Pathol Anat Histopathol** 1992; 421: 87-93.

Williams AF, Barclay AN: The immunoglobulin superfamily: Domains for cell surface recognition. **Annu Rev Immunol** 1988; 6: 381-405.

Winter G, Milstein C: Man-made antibodies. **Nature** 1991; 349: 293-299.

Wisse E, De Zanger RB, Charels K, et al: The liver sieve: Considerations concerning the structure and function of endothelial fenestrae, the sinusoidal wall and the space of Disse. **Hepatology** 1985; 5: 683-692.

Woodhouse CS, Morgan AC: Murine monoclonal IgG3 antibodies to human colorectal tumor-associated antigen: production and characterization of antibodies active in both antibody-dependent cellular cytotoxicity and complement-mediated cytolysis. **Cancer Res** 1989; 49: 2766-2772.

Yagel S, Feinmesser P, Waghorne C, et al: Evidence that β1-6 branched Asn-linked oligosaccharides on metastatic tumor cells facilitate invasion of basement membrane. **Int J Cancer** 1989; 44: 685-690.

Yamada N, Chung YS, Sawada T, et al: Role of SPan-1 antigen in adhesion of human colon cancer cells to vascular endothelium. **Dig Dis Sci** 1995; 40: 1005-1012.

Yee D, Cullen KJ, Paik S, et al: Insulin-like growth factor II mRNA expression in human breast cancer. **Cancer Res** 1988; 48: 6691-6696.

Yoneda J, Saiki I, Kobayashi H, et al: Inhibitory effect of recombinant fibronectin polypeptides on the adhesion of liver-metastatic lymphoma cell to hepatic sinusoidal endothelial cells and tumor invasion. **Jpn J Cancer Res** 1994; 85: 723-734.

Yoshimori T, Semba T, Takemoto H, et al: Protein disulfide-isomerase in rat pancreatic cells is exported from the endoplasmic reticulum despite possessing the retention signal. **J Biol Chem** 1990; 265: 15984-15990.

Zetter BR: The cellular basis of site - specific tumor metastasis. Seminars in Medicine of the Beth Israel Hospital, Boston, **N Eng J Med** 1990; 322: 605-612.

Zetter BR: Adhesion molecules in tumor metastasis. **Semin Cancer Biol** 1993;4: 219-229.

Zhu D, Cheng CF, Pauli BU: Mediation of lung metastasis of murine melanomas by a lung-specific endothelial cell adhesion molecule. **Proc Natl Acad Sci USA** 1991; 88: 9568-9572.

Zhu D, Cheng CF, Pauli BU: Blocking of lung endothelial cell adhesion molecule-1 (Lu-ECAM-1) inhibits murine melanoma lung metastasis. **J Clin Invest** 1992; 89: 1718-1724.

Zhu D, Pauli BU: Correlation between the lung distribution patterns of Lu-ECAM-1 and melanoma experimental metastases. **Int J Cancer** 1993; 53: 628-633.

Zola H: The surface antigens of human B lymphocytes. **Immunol Today** 1987; 8: 308-310.