EXHIBIT B

PART 1 OF 5

delivery of protein and peptide drugs in cancer

delivery of protein and peptide drugs in cancer

vladimir p torchilin
Northeastern University, USA

ICP Imperial College Press

Published by

Imperial College Press
57 Shelton Street
Covent Garden
London WC2H 9HE

Distributed by

World Scientific Publishing Co. Pte. Ltd.
5 Toh Tuck Link, Singapore 596224
USA office: 27 Warren Street, Suite 401-402, Hackensack, NJ 07601
UK office: 57 Shelton Street, Covent Garden, London WC2H 9HE

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library.

DELIVERY OF PROTEIN AND PEPTIDE DRUGS IN CANCER

ISBN 1-86094-627-5

Printed in Singapore by Mainland Press

Contents

Contributors xiii

1. Introduction 1

Vladimir Torchilin

References 6

2. Influence of Tumor Physiology on Delivery of Therapeutics 9

Robert B. Campbell

1. Introduction 9
2. Blood Vessels: Modulation of Normal and Pathologic Function 10
   - 2.1. General features of blood vessels in biological systems 10
   - 2.2. The tumor vasculature: Specialized features and angiogenesis 12
3. Transport of Peptide and Protein Molecules across Tumor Capillary Networks 14
   - 3.1. Barriers limiting drug transport 14

3.2. The interstitial matrix: MMPs, collagen, invasion and metastasis 19
3.3. Overcoming barriers: Exploiting tumor physiology for therapeutic gain 21
4. Concluding Remarks 29
References 29

3. Enhanced Permeability and Retention (EPR) Effect and Tumor-Selective Delivery of Anticancer Drugs **37**

Khaled Greish, Arun K. Iyer, Jun Fang, Michio Kawasuji and Hiroshi Maeda

1. Introduction 37
2. Theory Underlying the EPR Effect 38
3. Anatomical and Pathophysiological Abnormalities Related to EPR Effect 39
4. Augmentation of the EPR Effect in Solid Tumor by Influencing Vascular Mediators 42
5. Relation of Fluid Dynamics in Cancer Tissue to the EPR Effect 44
6. Implications for Delivery of Drugs to Tumors 45
7. Conclusion 48
References 48

4. Basic Strategies for PEGylation of Peptide and Protein Drugs **53**

Gianfranco Pasut, Margherita Morpurgo and Francesco M. Veronese

1. Introduction 54
2. Features of PEG as Bioconjugation Polymer 55
3. PEGylation Chemistry 61
   3.1. Amino group PEGylation 61
   3.2. Thiol PEGylation 67
   3.3. Carboxy PEGylation 68
4. Strategies in Protein PEGylation 69
5. Enzymatic Approach for Protein PEGylation 74
6. PEGylated Protein Purification and Characterization 76
7. Conclusion 78
References 79

5. PEGylated Proteins as Cancer Therapeutics 85

Margherita Morpurgo, Gianfranco Pasut and Francesco M. Veronese

1. Introduction 85
2. Enzymes and PEG-Enzymes in Cancer Therapy 87
   - 2.1. Metabolite depleting enzymes 88
     - 2.1.1. PEG-asparaginase (PEG-ASNase) 88
     - 2.1.2. PEG-methioninase (PEG-METase) 91
     - 2.1.3. PEG-arginine deiminase (PEG-ADI) 93
     - 2.1.4. PEG-uricase (PEG-UK) 95
3. PEG-Antibodies 100
4. Conclusions 103

References 104

6. PEGylated Proteins in Immunotherapy of Cancer 111

James F. Eliason

1. Introduction 112
2. PEGylated Interferon-α 114
3. PEGylated G-CSF 115
4. Other PEGylated Hematopoietic Growth Factors 117
5. PEGylated Lymphokines 118
6. PEGylated Cytokine Inhibitors 119
7. Conclusions and Outlook 119

References 120

7. Silencing Proteins: Nanotechnological Approaches to Deliver siRNA for Cancer Therapy 127

Raymond M. Schiffelers, Daan J.A. Crommelin and Gert Storm

1. Role of Proteins in Tumor Development 128
   - 1.1. Proto-oncogenes 128
   - 1.2. Tumor suppressor genes 128
   - 1.3. Pro-angiogenic proteins/pro-metastatic proteins 129
   - 1.4. Proteins involved in drug resistance 129
2. RNA Interference 129
   - 2.1. Mechanism of RNA interference 130
   - 2.2. Structural requirements for siRNA 131
3. Approaches for siRNA Delivery 134

4. siRNA Delivery for Cancer Therapy 135
4.1. siRNA to silence oncogenes 135
4.2. siRNA to silence mutated tumor suppressor genes . . . 138
4.3. siRNA to silence pro-angiogenic/ pro-metastatic genes 139
4.4. siRNA to silence genes that increase tumor drug resistance 141
5. Concluding Remarks 141
References 143

8. Anti-Cancer Proteins and Peptides in Liposomes 155

Vladimir Torchilin

1. Proteins and Peptides as Cancer Therapeutics 155
2. Stability, Delivery and Distribution Issues for Peptide and Protein Drugs 156
3. Possible Solutions. Liposomes as Pharmaceutical Carriers 158
4. Liposomal Proteins and Peptides for Cancer Treatment . . . 162
5. Tumor Cell Targeting Using Specific Ligand-Bearing Liposomes 165
6. Transmembrane Delivery of Protein and Peptide Drugs into Tumor Cells 167
References 171

9. Folate-Mediated Delivery of Protein and Peptide Drugs into Tumors 183

Joseph A. Reddy, Christopher P. Leamon and Philip S. Low

1. Introduction 184
2. Methods for Coupling FA to Proteins and Peptides 185
3. Cytotoxicity Studies of Folate Linked Toxins 188
4. Folate Targeted Immunotherapy 192
5. Folate Targeted Enzyme Prodrug Therapy 195
6. Folate-mediated Targeting of Adenovirus 195
7. *In vivo* Fate of Folate-protein Conjugates 197
8. Folate-targeted Peptidic Imaging Agent 198
9. Conclusions and Outlook 200
References 201

10. Transferrin Receptor Mediated Delivery of Protein and Peptide Drugs into Tumors **205**

Julia Fahrmeir and Manfred Ogris

1. Introduction . . . 205
2. Transferrin (Tf) . . . 206
   2.1. Structure . . . 206
   2.2. Biological function . . . 207
3. Transferrin-Receptors (TfR) . . . 207
   3.1. Structure . . . 207
   3.2. Clathrin-mediated endocytosis . . . 208
   3.3. Regulation of expression . . . 209
   3.4. Tissue distribution . . . 209
4. *In vivo* Application of Proteins Drugs, General Considerations . . . 210
5. Generation of Conjugates . . . 211
   5.1. Chemical coupling . . . 211
   5.2. Recombinant production of conjugates . . . 212
6. Tf and AntiTFR Targeted Toxins . . . 213
   6.1. Ribosomal inhibitors . . . 213
   6.2. Ribonucleases . . . 214
   6.3. Diphtheria toxin and Pseudomonas exotoxin . . . 215
7. Conclusions . . . 217

References . . . 217

11. Transmembrane Delivery of Protein and Peptide Drugs into Cancer Cells **225**

Cheryl C. Saenz and Steven F. Dowdy

1. Introduction . . . 226
2. Functional Characteristics of Protein Transduction Domains . . . 227
   2.1. TAT peptide . . . 227
   2.2. TAT-related peptides . . . 227
3. Mechanism of PTD-Mediated Transduction . . . 228
4. Delivery of Anti-Cancer Therapies Using Protein Transduction Domains . . . 229

4.1. Loss of tumor suppressor function 230
4.1.1. p53 tumor suppressor 230
4.1.2. p16 tumor suppressor 232
4.1.3. p21 and p27 tumor suppressors 232
4.1.4. VHL tumor suppressor 234
4.1.5. NF2/merlin tumor suppressor 234
4.2. Resistance to apoptosis 235
4.2.1. Caspase activation 235
4.2.2. Bcl-2 family proteins 237
4.2.3. S100 family proteins 237
4.2.4. Protein kinase 2 (casein kinase 2) 238
4.2.5. Activating transcription factor 2 238
4.3. Activation of oncogenes 239
4.3.1. Ras signaling pathways 239
4.3.2. HER-2 pathway 240
4.4. Resistance to conventional cancer therapeutics 241
4.4.1. RasGAP-derived peptide 241
4.4.2. Repair of mitochondrial DNA 242
4.5. Additional applications of PTD-mediated delivery in the treatment of cancer cells 243
4.5.1. Cancer cell vaccines 243
4.5.2. Cancer cell migration 244
5. Conclusions 245
References 245

12. Protein and Peptide Drugs to Suppress Tumor Angiogenesis 255

Curzio Rüegg

1. Introduction 255
2. Natural Cyokines 256
3. Cryptic Protein Fragments 262
4. Antibodies 264
4.1. Monoclonal antibodies (mAb) 264
4.2. Recombinant antibodies 267
5. Soluble VEGF-Rs (Traps) 269
6. Small Peptides 269
6.1. Vascular targeting peptides 269

6.2. Function-blocking peptides 271
7. Conclusions and Perspectives 273
References 273

13. Utilizing Lymphatic Transport in Enhancing the Delivery of Drugs, Including Proteins, and Peptides, to Metastatic Tumors **285**

Ellen K. Wasan and Kishor M. Wasan

1. Introduction 286
2. Lipid Absorption from the Small Intestine and the Lymphatic System 287
   2.1. Lipid absorption from the small intestine 287
   2.2. Intestinal lymphatic system 290
3. Lymphatic Vessels Functioning as Transport Routes for Malignant Cells 290
4. Biological and Pharmaceutical Factors Affecting Lymphatic Drug Delivery 291
   4.1. Contribution of lymphatic transport to the increased absorption of water-insoluble drugs into the systemic circulation 291
   4.2. Evaluation and assessment of intestinal lymphatic transport 293
   4.3. Proposed mechanisms that govern the lymphatic transport of water-insoluble drugs 295
      4.3.1. Diffusion and partition behavior of water-insoluble drugs 295
      4.3.2. Lipid solubility of water-insoluble drugs 296
5. Utilization of Lymphatic System in the Delivery of Drugs, Including Proteins and Peptides to Metastatic Tumors . . . 297
6. Clinical Significance 300
References 301

14. Delivery of Protein and Peptide Drugs to Brain Tumors **309**

Herbert B. Newton

1. Introduction 310
2. Biology of the BBB, BBB-Transport Systems, and BTB 311

3. Overview of Drug Delivery and Distribution 314
4. Chemical Modification, Glycosylation, Pegylation, and Other Delivery Approaches 315
5. Nanoparticle Drug Delivery Approaches 318
6. Liposomal Drug Delivery Approaches 320
7. Convection-Enhanced Delivery Approaches 323
8. Immunoglobulin-Based Delivery Approaches 323
9. Vector-Mediated Delivery Approaches 326
References 328

15. Protein and Peptide-Based Cancer Gene Therapy 335

Sunil Chada and Rajagopal Ramesh

1. Cancer Gene Therapy 335
   1.1. Identifying the target gene/pathway for cancer therapy 336
   1.2. Selection of gene therapy vector 338
2. Protein-Based Cancer Gene Therapy 339
   2.1. Growth inhibition and apoptosis 340
      2.1.1. Toxic proteins 340
      2.1.2. Tumor suppressor proteins 343
      2.1.3. Antiangiogenic proteins 348
   2.2. Enhancement of the immune response 351
3. Peptide-Based Cancer Gene Therapy 353
   3.1. Tumor targeting and gene transduction 353
4. Conclusions 357
References 357

Index 371

Contributors

Robert B Campbell
Department of Pharmaceutical Sciences,
Northeastern University,
360 Huntington Ave
Bouvé College of
Health Sciences,
110 Mugar Hall
Boston, MA 02115,
USA

Sunil Chada
Introgen Therapeutics, Inc.,
2250 Holcombe Blvd.,
Houston, TX 77030,
USA

Daan J A Crommelin
Department of Pharmaceutics,
Utrecht Institute for
Pharmaceutical Sciences,
Utrecht University,
Utrecht,
The Netherlands

Steven F Dowdy
Howard Hughes Medical Institute,
and Department
of Cellular and Molecular Medicine,
George Palade Laboratories,
Room 231B,
USCD School of Medicine,
9500 Gilman Drive,
Dept. 0686,
La Jolla, CA 92093-0686

James F Eliason
Asterand, Inc.,
TechOne Suite 501,
440 Burroughs,
Detroit, MI 48202-3420
USA

Julia Fahrmeir
Pharmaceutical
Biology-Biotechnology,
Center of Drug Research
Department of Pharmacy,
Butenandtstrasse 5-13,
81377 München,
Germany

Jun Fang
Faculty of Pharmaceutical
Sciences, Sojo University,
4-22-1, Ikeda Kumamoto,
860-0082, Japan

Khaled Greish
Faculty of Pharmaceutical
Sciences, Sojo University,
4-22-1, Ikeda, Kumamoto,
860-0082, Japan

Arun K Iyer
Faculty of Pharmaceutical
Sciences, Sojo University,
4-22-1, Ikeda, Kumamoto,
860-0082, Japan

Michio Kawasuji
Department of Cardiovascular
Surgery, Kumamoto
University Graduate
School of Medical Sciences,
Honjo 1-1-1, Kumamoto, Japan

Christopher P Leamon
Endocyte, Inc.,
3000 Kent Avenue,
Suite A1-100
West Lafayette,
IN 47906, USA

Philip S Low
Department of Chemistry,
Purdue University,
560 Oval Drive
West Lafayette,
IN 47906, USA

Hiroshi Maeda
Faculty of Pharmaceutical
Sciences, Sojo University,
4-22-1, Ikeda, Kumamoto,
860-0082, Japan

Margherita Morpurgo
Department of
Pharmaceutical Science,
University of Padova,
via F. Marzolo 5,
35131-Padova,
Italy

Herbert B Newton
Dardinger Neuro-Oncology
Center and Division
of Neuro-Oncology,
Departments of Neurology
and Oncology,
Ohio State
University Medical
Center and James
Cancer Hospital
465 Means Hall
1654 Upham Drive,
Columbus,
Ohio 43210, USA

Manfred Ogris
Pharmaceutical
Biology-Biotechnology,
Center of Drug Research
Department of Pharmacy,
Butenandtstrasse 5-13 (Haus D)
81377 München
Germany

Gianfranco Pasut
Department of
Pharmaceutical Science,
University of Padova,
via F. Marzolo 5,
35131-Padova,
Italy

Rajagopal Ramesh
Department of Thoracic
and Cardiovascular
Surgery, Unit 445
M.D. Anderson Cancer Center,
P.O. Box 301402
1515 Holcombe Blvd
Houston, TX 77230-1402, USA

Joseph A Reddy
Endocyte, Inc.,
3000 Kent Avenue, Suite A1-100,
West Lafayette,
IN 47906, USA

Curzio Rüegg
Lausanne Cancer Center (LCC)
Swiss Institute for
Experimental Cancer
Research (ISREC)
Chemin des Boveresses 155
CH-1066 Epalinges
Switzerland

Cheryl C Saenz
Department of
Reproductive Medicine,
USCD School of Medicine,
La Jolla, California
92093-8433, USA

Raymond M Schiffelers
Department of Pharmaceutics,
Utrecht Institute for
Pharmaceutical Sciences,
PO Box 80.082,
3508 TB Utrecht,
The Netherlands

Gert Storm
Department of Pharmaceutics,
Utrecht Institute for
Pharmaceutical Sciences,
Utrecht University,
Utrecht,
The Netherlands

Vladimir Torchilin
Department of Pharmaceutical
Science, Bouvé College
of Health Sciences,
Northeastern University
312 Mugar Hall,
Boston,
MA 02115,
USA

Francesco M Veronese
Department of
Pharmaceutical Science,
University of Padova,
via F. Marzolo 5,
35131-Padova
Italy

Ellen K Wasan
Advanced Therapeutics,
Faculty of Pharmaceutical Sciences,
University of British Columbia,
BC Cancer Agency
675 West 10th Ave
Vancouver, BC V5Z 1L3
Canada

Kishor M Wasan
Division of Pharmaceutics
and Biopharmaceutics,
Faculty of Pharmaceutical Sciences,
University of British Columbia,
2146 East Mall
Vancouver, British Columbia
Canada V6T 1Z3

1

Introduction

Vladimir Torchilin

To effectively treat cancer, we have to be able to selectively attack the tumor and individual cancer cells, while effectively protect normal tissue from possible toxicity and other side effects of anti cancer drugs. It is not an easy task, since systemically administered drugs may rapidly metabolize in the blood or be cleared from the body before reaching the tumor cells. In addition, on its way to the target, it has to overcome multiple physiological barriers, such as irregularities in the tumor blood flow, the high interstitial pressure, and the absence of a lymphatic drainage in tumors (Campbell, Chapter 2). Many drugs have also been found to perform their action inside the cells which requires their intracellular delivery through low permeable cell membranes. All these obstacles are especially pronounced in the case of protein and peptide drugs, whose successful application needs effective means of drug delivery into tumors. This book will consider various problems associated with tumor delivery of protein and peptide drugs and some of the current strategies to solve these problems.

It is well known that any proteins and peptides possess biological activity that makes them therapeutically potent, in particular, anticancer agents. Advances in solid-phase peptide synthesis and recombinant DNA and hybridoma technology allow for production of unlimited quantities of clinical grade protein and peptides. The use of proteins and peptides

as therapeutic agents is hampered, however, by their fast elimination from circulation mostly because of renal filtration, fast enzymatic degradation, uptake by the reticuloendothelial system (RES) and accumulation in non-targeted organs and tissues. Fast elimination and distribution into non-targeted organs and tissues cause the need to administer a drug in large quantities, which is often uneconomical and sometimes impossible due to non-specific toxicity. Low permeability of cell membranes for macromolecules often represents an additional obstacle for the development of protein and peptide based anticancer formulations. Numerous approaches to overcome fast elimination and non-specific biodistribution of conventional drugs have been developed and can be adapted for the delivery of anticancer protein and peptides. This book focuses on injectable microscopic systems for the delivery of protein and peptide anticancer agents to and into tumors. Main advantages of these systems over macroscopic devices include greater convenience and less invasive administration, the ability to reach delocalized targets and a lower manufacturing cost.

One of the reasons for fast clearance from systemic circulation of proteins and peptides with molecular weight of 40 kDa or lower is renal filtration. This issue may be addressed by conjugation of the biomolecules with water-soluble polymers, which results in a complex with high enough molecular weight.[1] Additional benefits of protein (peptide)–polymer conjugation are increased resistance against enzyme degradation and lowered immunogenicity. Both enzymatic degradation and immune response against a protein cause its fast elimination from the systemic circulation. The developing of the immune response, in addition, is potentially dangerous because of the possibility of allergic reactions and anaphylactic shock upon repetitive administrations. Polymer molecules attached to the protein globule create steric hindrances, which interfere with active sites of proteases,[1] opsonins or antigen-processing cell.[2] Currently, poly(ethylene glycol) (PEG) is the most popular polymer for modification of proteins with therapeutic potential[3–5] (Veronese, Chapter 4; Eliason, Chapter 6). PEG modified L-asparaginase has been proposed as an anticancer agent as early as in 1984.[5] This formulation (Oncospar® from Enzon) was approved as an orphan drug in the US for use in lymphoma and leukemia treatments.[6] It has a longer circulation time than the original enzyme and does not induce hypersensitivity reaction in patients with such reaction to the non-modified enzyme.[7,8] In some cases, drugs are conjugated with polymers that can

attach themselves and conjugated drug to natural long-circulating blood plasma components, like serum albumin or lipoproteins. Thus, the conjugation of proteins and peptides with poly(styrene-co-maleic acid/anhydride) (SMA)[9] increases the circulation time of anticancer proteins and peptides *via* the binding of the conjugates to plasma albumin.[10] The conjugation with SMA also protects proteins from enzymatic degradation, and decreases immunogenicity of modified proteins.[9] SMA-modified neocarzinostatin is currently approved in Japan for hepatoma treatment.[11]

High molecular weight (40 kDa or higher), long-circulating macromolecules, including proteins and peptides conjugated with water-soluble polymers, are capable of spontaneous accumulations in solid tumors via the enhanced permeability and retention effect (EPR).[9,11] This effect is based on the fact that tumor vasculature, unlike vasculature of healthy tissues, is "leaky", i.e. penetrable for macromolecules and nanoparticulates, which allows macromolecules to accumulate in the interstitial tumor space (see Maeda, Chapter 3). Such accumulation is also facilitated by the fact that lymphatic system, responsible for the drainage of macromolecules from normal tissues, is virtually not working in case of many tumors as a result of the disease.[11]

Nanoparticulate drug delivery systems may represent a valid alternative to soluble polymeric carriers. This type of systems includes liposomes, micelles, polymer microparticles, etc. The use of this type of carriers allows achieving much higher active moiety/carrier material ratio compared with "direct" molecular conjugates. They also provide better protection of protein and peptide drugs against enzymatic degradation and other destructive factors upon parenteral administration because the carrier wall completely isolates drug molecules from the environment. All nanoopariculate carriers have the size, which excludes a possibility of renal filtration. The main disadvantage of microreservoir carriers is their tendencies to be taken up by the RES cells primary in liver and spleen.[12] Among particulate drug carriers, liposomes are the most extensively studied and poses the most suitable characteristics for protein (peptide) encapsulation (Torchilin, Chapter 8). Similar to macromolecules, liposomes are capable of accumulating in tumors of various origins *via* the EPR effect.[13,14] In some cases, however, the liposome size is too large to provide an efficient accumulation via the EPR effect presumably due to relatively small tumor vasculature cut off size.[15,16] In such cases, alternative delivery systems with smaller sizes

such as micelles (prepared, for example, from PEG-phospholipid conjugates) can be used. These particles lack the internal aqueous space and are smaller than liposomes. Protein or peptide pharmaceutical agent can be covalently attached to the surface of these particles or incorporated into them via chemically attached hydrophobic group ("anchor").[15]

The use of vector molecules can further enhance tumor targeting of protein/peptide drugs or protein/peptide-loaded nanocarries or make them EPR effect independent. The latter is especially important for the cases of tumors with immature vasculature, such as tumors on the earlier stages of their development, and delocalized tumors. Vector molecules (those having affinity toward ligands characteristic for target tissues) capable of recognizing tumors were found among antibodies, peptides, lectines, saccharides, hormones, and some low molecular weight compounds[17] (Reddy, Chapter 9; Ogris, Chapter 10). From this list, antibodies and their fragments provide the most universal opportunity to target various targets and have the highest potential specificity. Antibodies capable of recognizing specific antigens were derived for the majority of known tumors.[18] Recent advances in recombinant engineering make it possible to produce anticancer antibodies on industrial scale at relatively low cost. Humanized versions of antibodies and their fragments in which rodent-derived binding sites and human conservative regions are combined using recombinant technology became available.[19,20]

The successful delivery of anticancer drugs, proteins and peptides among them, into tumors does, however, solve only a part of a general efficiency problem. The following task is to achieve their intracellular delivery, since many targets for anticancer drugs are located inside cells (e.g. the surface of mitochondria may serve as a promising target for apoptosis-inducing drugs). A huge body of available information about cellular metabolic and signaling pathways essential for tumorogenesis and tumor cell development allows for identifying protein targets for interference with the tumor growth. Quite a few molecular targets have already been identified.[21] The creation of a working draft of the human genome sequence[22,23] in combination with high-throughput methods of molecular biology promises continued rapid growth in identifying such targets.[24,25] Sometimes tumor results from the malfunctions of tumor suppressor genes, as well as the lack of activity of the proteins they encode.[25,26] In this case, the delivery into tumor cells of working copies of proteins obtained by

recombinant methods would provide indispensable tools for the validation of gene functions and potential development of protein or gene therapy-based methods of treatment (Chada, Chapter 15; Crommelin, Chapter 7).

Generally, the use of peptides and proteins for molecular target validation and eventual development of anticancer drugs is hampered by the low permeability of cell membranes. The very nature of cell membranes prevents protein/peptide entering unless there is an active transport mechanism, which is usually the case for very short peptides.[27] As mentioned above, vector molecules promote the delivery of associated drug-carriers inside the cells via receptor-mediated endocytosis.[28] An efficient cellular uptake via endocytosis is generally observed, but the delivery of intact proteins and peptides is compromised by an insufficient endosomal escape and lysosomal degradation. An enhanced endosomal escape can be achieved through the use of, for example, lytic peptides,[29,30] pH-sensitive polymers[31] or swellable dendritic polymers.[32] Although these agents have provided encouraging results in overcoming limitations of endocytosis-based cytoplasmic delivery, there is still a need for further improvements or alternative delivery strategies. An approach recently emerged, which provides for a much more straightforward and efficient way for delivery of proteins and peptides to the cytoplasm. This approach is based on the phenomenon called transduction (Dowdy, Chapter 11), and uses the ability of certain peptides to ferry conjugated macromolecules, such as proteins[33] and DNA, and even particles as large as 40 nm iron oxide colloidal particles[34,35] and 200 nm liposomes,[36,37] across cell membranes directly into cytoplasm. Peptides that cause transduction (PTDs, protein transduction domains, or CPPs, cell-penetrating peptides) can be as short as 10-to-16-mer.[33,38,39] Several proteins including those involved in oncogenesis, cancer-related signal transductuction and cell proliferation pathways have been delivered in active form into various human cells *in vitro* using fused PTD peptides.[40–43] It has also been shown that TAT PTD allows delivery of biologically active proteins into various cells *in vivo*.[44] These results open new avenues in the development of protein and peptide-based anticancer therapeutics with intracellular molecular targets.

Thus, current knowledge provides some promising approaches how to deliver protein and/or peptide-based anticancer drugs into tumors (see also Wasan, Chapter 13) and further inside tumor cells. This opens new opportunities for improved therapy of various cancers (see Ruegg,

Chapter 12, Newton, Chapter 14). This book certainly covers only a fraction of issues related to the use of protein and peptide drugs in cancer therapy. Still, we hope that the information it contains will be useful for academics and clinicians involved in related research.

References

1. Torchilin VP (1991) Immobilized enzymes in medicine. *Progress in Clinical Biochemistry and Medicine*. **11**:206, Heildelberg Springer-Verlag: Berlin.
2. Harris JM, Martin NE and Modi M (2001) Pegylation: A novel process for modifying pharmacokinetics. *Clin Pharmacokinet* **40**:539.
3. Veronese FM and Harris JM (2002) Introduction and overview of peptide and protein pegylation. *Adv Drug Deliv Rev* **54**:453.
4. Roberts MJ, Bentley MD and Harris JM (2002) Chemistry for peptide and protein PEGylation. *Adv Drug Deliv Rev* **54**:459.
5. Abuchowski A, Kazo GM, Jr, Verhoest CR, Van Es T, Kafkewitz D, Nucci ML, Viau AT and Davis FF (1984) Cancer therapy with chemically modified enzymes. I. Antitumor properties of polyethylene glycol-asparaginase conjugates. *Cancer Biochem Biophys* **7**:175.
6. Ettinger AR (1995) Pegaspargase (Oncaspar), *J Pediatr Oncol Nurs* **12**:46.
7. Asselin BL (1999) The three asparaginases. Comparative pharmacology and optimal use in childhood leukemia. *Adv Exp Med Biol* **457**:621.
8. Holle LM (1997) Pegaspargase: An alternative? *Ann Pharmacother* **31**:616.
9. Maeda H (2001) SMANCS and polymer-conjugated macromolecular drugs: Advantages in cancer chemotherapy. *Adv Drug Deliv Rev* **46**:169.
10. Mu Y, Kamada H, Kaneda Y, Yamamoto Y, Kodaira H, Tsunoda S, Tsutsumi Y, Maeda M, Kawasaki K, Nomizu M, Yamada Y and Mayumi T (1999) Bioconjugation of laminin peptide YIGSR with poly(styrene co-maleic acid) increases its antimetastatic effect on lung metastasis of B16-BL6 melanoma cells. *Biochem Biophys Res Commun* **255**:75.
11. Maeda H, Sawa T and Konno T (2001) Mechanism of tumor-targeted delivery of macromolecular drugs, including the EPR effect in solid tumor and clinical overview of the prototype polymeric drug SMANCS. *J Control Rel* **74**:47.
12. Senior H (1987) Fate and behavior of liposomes in vivo: A review of controlling factors. *Crit Rev Ther Drug Carrier Syst* **3**:123.
13. Yuan F, Leunig M, Huang SK, Berk DA, Papahadjopoulos D and Jain RK (1994) Microvascular permeability and interstitial penetration of sterically stabilized (stealth) liposomes in a human tumor xenograft. *Cancer Res* **54**:3352.

14. Gabizon AA (2001) Pegylated liposomal doxorubicin: Metamorphosis of an old drug into a new form of chemotherapy. *Cancer Invest* **19**:424.
15. Weissig V, Whiteman KR and Torchilin VP (1998) Accumulation of protein-loaded long-circulating micelles and liposomes in subcutaneous Lewis lung carcinoma in mice. *Pharm Res* **15**:1552.
16. Hobbs SK, Monsky WL, Yuan F, Roberts WG, Griffith L, Torchilin VP and Jain RJ (1998) Regulation of transport pathways in tumor vessels: Role of tumor type and microenvironment. *Proc Natl Acad Sci USA* **95**:4607.
17. Gregoriadis G (1977) Targeting of drugs. *Nature* **265**:407.
18. Torchilin VP, Ed. (1995) *Handbook of Targeted Delivery of Imaging Agents*. CRS Press, Boca Raton.
19. Jurcic JD, Scheinberg DA and Houghton AN (1997) Monoclonal antibody therapy of cancer. *Cancer Chemother Biol Response Modif* **17**:195.
20. Dillman RO (2001) Monoclonal antibodies in the treatment of malignancy: Basic concepts and recent developments. *Cancer Invest* **19**:833.
21. Gibbs JB (2001) Mechanism-based target identification and drug discovery in cancer research. *Science* **287**:1969.
22. Venter JC, *et al.* (2001) The sequence of the human genome. *Science* **291**:1304.
23. Lander ES, *et al.* (2001) Initial sequencing and analysis of the human genome. *Nature* **409**:860.
24. Workman P (2001) New drug targets for genomic cancer therapy: Successes, limitations, opportunities and future challenges. *Curr Cancer Drug Targets* **1**:33.
25. Balmain A (2001) Cancer genetics: From Boveri and Mendel to microarrays. *Nat Rev Cancer* **1**:77.
26. Hussain SP, Hofseth LJ and Harris CC (2001) Tumor suppressor genes: At the crossroads of molecular carcinogenesis, molecular epidemiology and human risk assessment. *Lung Cancer* **34**(Suppl 2):S7.
27. Egleton RD and Davis TP (1997) Bioavailability and transport of peptides and peptide drugs into the brain. *Peptides* **18**:1431.
28. Park JW, Kirpotin DB, Hong K, Shalaby R, Shao Y, Nielsen UB, Marks JD, Papahadjopoulos D and Benz CC (2001) Tumor targeting using anti-her2 immunoliposomes. *J Control Rel* **74**:95.
29. Kamata H, Yagisawa H, Takahashi S and Hirata H (1994) Amphiphilic peptides enhance the efficiency of liposome-mediated DNA transfection. *Nucleic Acids Res* **22**:536.
30. Midoux P, Kichler A, Boutin V, Maurizot JC and Monsigny M (1998) Membrane permeabilization and efficient gene transfer by a peptide containing several histidines. *Bioconjug Chem* **9**:260.

31. Lackey CA, Press OW, Hoffman AS and Stayton PS (2002) A biomimetic pH-responsive polymer directs endosomal release and intracellular delivery of an endocytosed antibody complex. *Bioconjug Chem* **13**:996.
32. Padilla De Jesus OL, Ihre HR, Gagne L, Frechet JM and Szoka FC Jr (2002) Polyester dendritic systems for drug delivery applications: In vitro and in vivo evaluation. *Bioconjug Chem* **13**:453.
33. Vives EP, Brodin P and Lebleu B (1997) A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J Biol Chem* **272**:16010.
34. Lewin M (2000) Tat peptide-derivatized magnetic nanoparticles allow in vivo tracking and recovery of progenitor cells. *Nat Biotechnol* **18**:410.
35. Bhorade R (2000) Macrocyclic chelators with paramagnetic cations are internalized into mammalian cells via a HIV-tat derived membrane translocation peptide. *Bioconjug Chem* **11**:301.
36. Torchilin VP, Rammohan R, Weissig V and Levchenko TS (2001) TAT peptide on the surface of liposomes affords their efficient intracellular delivery even at low temperature and in the presence of metabolic inhibitors. *Proc Natl Acad Sci USA* **98**:8786.
37. Tseng YL, Liu JJ and Hong RL (2002) Translocation of liposomes into cancer cells by cell-penetrating peptides penetratin and tat: A kinetic and efficacy study. *Mol Pharmacol* **62**:864.
38. Derossi D, Joliot AH, Chassaing G and Prochiantz A (1994) The third helix of the Antennapedia homeodomain translocates through biological membranes. *J Biol Chem* **269**:10444.
39. Elliott G and O'Hare P (1997) Intercellular trafficking and protein delivery by a herpesvirus structural protein. *Cell* **88**:223.
40. Vocero-Akbani A, Lissy NA and Dowdy SF (2000). Transduction of full-length Tat fusion proteins directly into mammalian cells: Analysis of T cell receptor activation-induced cell death. *Methods Enzymol* **322**:508.
41. Soga N, Namba N, McAllister S, Cornelius L, Teitelbaum SL, Dowdy SF, Kawamura J and Hruska KA (2001) Rho family GTPases regulate VEGF-stimulated endothelial cell motility. *Exp Cell Res* **269**:73.
42. Zezula J, Casaccia-Bonnefil P, Ezhevsky SA, Osterhout DJ, Levine JM, Dowdy SF, Chao MV and Koff A (2001) p21cip1 is required for the differentiation of oligodendrocytes independently of cell cycle withdrawal. *EMBO Rep* **2**:27.
43. Hsia CY, Cheng S, Owyang AM, Dowdy SF and Liou HC (2002) c-Rel regulation of the cell cycle in primary mouse B lymphocytes. *Int Immunol* **14**:905.
44. Schwarze SR, Ho A, Vocero-Akbani A and Dowdy SF (1999) *In vivo* protein transduction: Delivery of a biologically active protein into the mouse. *Science* **285**:1569.

2

Influence of Tumor Physiology on Delivery of Therapeutics

Robert B. Campbell

1. Introduction

The concept of delivering therapeutic peptides and larger sized proteins to tumors has developed rapidly over the last few decades. The goal is to maximize delivery of therapeutics to tumor targets while minimizing effects on healthy organ tissues. Current approaches aim to selectively target cancer cells that have invaded host tissues, or to attack tumor vessels in order to arrest neovascularization or abolish mature vascular function. In view of spectacular advances, it is no surprise that drug delivery has achieved such prominence and has now emerged at the forefront of biomedical research and in many clinical environments.

It is important that investigators developing new treatment approaches against cancer both understand and safeguard against the many barriers impeding the optimal delivery of peptides, proteins and other therapeutics to solid tumors. In this chapter, we highlight the obstacles confronted today by drug delivery experts in their efforts to streamline global research in the fight against cancer and progression of disease. More specifically, we discuss the physiology of tumors in terms of the structure and function of vessels in normal and tumor tissues and in exploitable tumor targets to

improve drug-target recognition. The role of physiological factors will be evaluated, including ways to exploit specialized features of solid tumors and reduce the influence they have on drug delivery and transport.

2. Blood Vessels: Modulation of Normal and Pathologic Function

2.1. *General features of blood vessels in biological systems*

The endothelium is a structural barrier separating the intravascular compartment from the interstitial environment. Because of wide variability in anatomical structure, the vascular compartment is further grouped into three different subcategories. The subcategories are continuous, fenestrated or discontinuous endothelia.

Continuous endothelia are the most common type and found most frequently in blood vessels lining chambers of the heart, walls of capillaries and arterioles in skeletal, skin, cardiac muscle, and connective tissue and are well known for their relatively tight cellular junctions.[1,2] This particular category of vessels is also critical in the regulation and rapid exchange of ions and solutes.[1,2] Plasmalemmal vesicles involved in endothelial transport are abundant in myocardial endothelia but are far less frequently observed in capillaries of the brain.[3] The actual number of plasmalemmal vesicles existing along the continuous endothelium is thus heterogeneous, varying as a function of organ and tissue environment. These vesicular structures are also highly sensitive to charge characteristics, favoring associations with anionic over cationic proteins and other small circulating molecules.[4]

Fenestrated vessels are normally found in vessels of organs that secrete (or excrete) biological fluids as in the gastrointestinal mucosa and in the glomerular capillaries of the kidneys. Fenestrae are usually between 50 and 80 nm in size and appear either as individual gap openings in the wall of functional vessels or as clusters. Similar to plasmalemmal vesicles, their frequency of occurrence along vessels depends on organ type and microenvironment.

Often two or more capillaries may join to form post-capillary venules. These newly formed networks are composed of a single lining of endothelial cells with a basement membrane with no smooth muscle cell attachment. These vessels are heavily involved in exchange of molecules and are

preferential sites of plasma extravasation as a result of the actions of vasoactive and humoral factors. Fenestrae possess a negatively charged surface density due to high heparin sulfate proteoglycan content, and unlike plasmalemmal vesicles of continuous endothelia, they favor interaction with cationic over anionic molecules.[5]

Discontinuous endothelia are found primarily in the liver, spleen and bone marrow organs.[1] In the liver sinusoids, the endothelia are not continuous and possess an average fenestrae size between 100 and 150 nm in diameter, with the size of the fenestrate often changing in response to local mediators. These changes include, but are not limited to, response to luminal pressures and potent vasodilators such as histamine and bradykinin.[6–9] An investigation into the size of vascular pore openings of tumors revealed gap openings that are significantly larger than those observed along vessels in normal tissues, around 4 microns (4000 nm) in at least one tumor type, but normally falling within the range of 0.4 to 0.6 microns (400 to 600 nm) in others studied.[10,11] Nonetheless, the evidence is overwhelmingly in favor of the development of tumor targeted delivery of therapeutic carrier molecules that are small enough to enter through tumor vascular pores without passing through openings in normal healthy organ tissues.

The endothelium is responsible for synthesizing a variety of molecules regulating endothelial cell migration, proliferation, blood vessel maturation and function. It has been shown to synthesize vascular growth factors, nitric oxide, collagen IV, laminin, glycosaminoglycans and proteoglycans to highlight several proven functions.[9,12–18] The physical barrier organizes very rapidly to form monolayers, reassembles to form vascular tubes,[19] and can change specific inter- and intracellular signaling patterns to meet highly specialized needs of the host. Additionally, endogenous and exogenous mediators of immune and inflammatory response regulate specialized functions at the surface of the endothelium. The endothelium can thus be considered an effective mediator of organ homeostasis.[9,20]

In many ways, the vascular networks found in solid tumors poorly resemble the more regular, well-defined vascular structure observed in disease-free tissues. Tumors, for example, have a highly chaotic arrangement of vessels compared with vessels in normal tissues. Tumor vessels also have an overabundance of anionic phospholipids in addition to a number of other negatively charged functional groups.[21–24] In view of the negatively charged molecules, glycosaminoglycans carry out important functions in

the metastatic disease process, and much like phospholipids, can serve as useful targets of peptide and protein therapeutics. Evaluation of altered proteoglycan expression in human breast tissue revealed a total proteoglycan content that was significantly increased in comparison with that in healthy tissues.[25] Proteoglycans isolated from malignant breast tissue have been shown to stimulate endothelial cell proliferation, and the total glycoprotein content in tumors is produced by many cell types, including cancer and tumor endothelial cells alike.

The vascular networks of tumors have an increased permeability for macromolecules and a higher proliferation rate of endothelial cells compared with vessels in quiescent tissues.[26,27] An estimated 30- to 40-fold increase in the growth rate of endothelial cells lining vessels in tumors compared with that in normal tissues has been demonstrated.[26]

Direct access to intravenously administered agents, rapid proliferation rate of endothelial cells and over-expression of negatively charged functional groups along vessels are potentially exploitable features of tumors. Peptide-free or endothelium-specific drug carrier molecules conjugated to potent peptide therapeutics can impede tumor growth on molecular and pharmacological levels.

2.2. *The tumor vasculature: Specialized features and angiogenesis*

The basic structure of a solid tumor, including the existence of its blood supply and some other important structural-related features, were first discovered by Rudolph Virchow during the 1860s.[28] During the early 1900s, Goldman[29] investigated the increased vascular supply in malignant diseases and the disorganized growth patterns of tumor vessels. Roughly 40 years later, it was confirmed that the growth of a transplanted tumor was connected to its ability to induce continuous endothelial cell growth.[30] The most relevant contribution to the study of *tumor vasculature* was made around the early 1970s when Gimbrone and Folkman first discovered that solid tumors require the development of new blood vessels to reach maturity, and that when tumor vascular growth was prevented, tumor dormancy was observed.[31,32] Today, nearly 35 years later the vast majority of new treatments are developed with the understanding that neovascularization is absolutely essential for malignant transformation.

Under normal circumstances the process of forming new blood vessels from pre-existing vessel networks (aka ~ angiogenesis) is observed during embryonic development and wound healing.[31] Angiogenesis is more commonly associated with pathological diseases involved in tissue regeneration; some other conditions include diabetic retinopathy, rheumatoid arthritis, chronic inflammatory diseases and cancer.[33] Microvascular networks are a vital component in the development of solid tumors. Efficient gas exchange, waste removal and delivery of nutrients to tissue-invading cancer cells depend on angiogenesis, without which the maximum size a tumor can reach is ~1–2 mm.[31] Until a new blood supply is recruited, tumors obtain the oxygen and nutrients they need for survival through passive diffusion. Once angiogenesis has begun, it remains as an active part of a tumor's life. Angiogenesis is not very active near the center of the tumor, but is a routine and highly efficient process near the tumor periphery. Given that angiogenesis does not occur with the same efficiency in all tumor regions, peptide and protein based therapeutics should be applied accordingly.

As tumors develop, a region deprived of oxygen and nutrients near the center of the tumor is formed. Many cells then die due to the severe hypoxic conditions. Tumor ischemic necrosis is therefore apparent in many solid tumors. Hypoxic conditions are probably linked to an insufficient number of blood vessels that undoubtedly influence the cells belonging to this hostile environment. For this reason, differential growth kinetics exists between cancer cells in well-oxygenated tumor regions, and neoplastic cells in regions that possess an inadequate blood supply. Assuming both cellular populations have found a way to adapt to their respective environments, all cells of a particular tumor region must thus share region-specific cell survival mechanisms that ensure adaptation of cells to a particular environment. Regardless of the tumor regions selecting for particular cellular characteristics (or expressed features), angiogenesis is absolutely essential for sustaining and maintaining the life of all solid tumors. Furthermore, the endothelial cells recruited by a developing tumor mass during angiogenesis are useful targets of peptide and protein therapeutics.[34,35]

As a tumor grows, it soon develops a nutrient-deprived tumor center. The poor-nutrient environment leads to dead cells due to hypoxia. Tumor vessels are either recruited from pre-existing vessels of the host or develop as a result of neovascularization.[31] Small venules and capillaries are involved in these processes. The formation of arteries and

arterioles in tumors and the invasion of cancer cells in these vascular types are not unprecedented, but rarely observed. Furthermore, vessels possessing layers of smooth muscle do not respond to the instructional call of proangiogenic stimuli, i.e. vascular endothelial growth factor (VEGF), to form new vessels. Regardless of the angiogenic stimulus and the mechanism(s) corresponding to the initiation signaling event, some important steps are commonly associated with the development of a new blood vessel. First, once an angiogenic stimulus has been recognized by the recruited venule or capillary, the basement membrane surrounding this vessel begins to degrade. Ausprunk and Folkman confirmed that this process is controlled by endothelial cells, and that the migration of endothelial cells approaches from the direction of the host vessel to the tumor.[36] A new capillary sprout next forms through openings created in the basement membrane.[36,37] The cytoskeleton of endothelial cells (lining the sprout) begins to curve and a lumen is formed. This event was first witnessed *in vivo* and later longitudinal vacuole formation was demonstrated in cultures of endothelial cells derived from bovine and human tissues.[19] The exact location and orientation of each endothelial cell with respect to the developing sprout will determine its overall role in this process. In general, the endothelial cells located at the tip and middle sections of the sprout perform highly specialized roles related to cell migration and mitosis, respectively. Once an individual sprout has formed, the same process continues to occur elsewhere in the vicinity of the new sprout, and two or more sprouts will frequently join together. Blood flows through the lumen; pericytes (or mural cells) arrange along the sprout; and a new basement membrane is constructed soon after the lumen forms.[38,39] The more often this process occurs, the more likely the tumor expands its vascular bed and increases in overall size.

3. Transport of Peptide and Protein Molecules across Tumor Capillary Networks

3.1. *Barriers limiting drug transport*

Suboptimal expression of tumor-associated antigens, multi-drug resistance, and insufficient binding of therapeutics to intended cellular targets cannot explain all the problems associated with delivery of therapeutics to solid tumors. It is generally understood that several physiological barriers also contribute to this problem. These barriers collectively represent some of the most serious issues facing formulation experts today.

The first barrier is the structural arrangement of tumor vessels. Vascular networks of tumors are structurally and functionally unique. The formation of loops and trifurcations clearly distinguish them from vessels in normal tissues.[29,38,40,41] There is insufficient evidence to relate the average number of vessels in a tumor to its size. Some reports suggest a constant vascular fraction over the lifespan of a tumor, while others report an actual reduction in vascular volume over a similar period.[42] Other factors include tumor type and microenvironment. This barrier is important given that the total vascular volume, the organization of tumor vessels, and irregular blood flow velocities all contribute as one unit to limit optimal delivery of macromolecules.

The second barrier involves issues related to tumor vascular permeability. Permeability to therapeutics is also a function of tumor type and anatomical location, and tumor vessels are therefore heterogeneous in terms of their permeability to circulating therapies.[10,11,22] In this regard, it is not uncommon to observe two tumor vessels in different microenvironments (or two vessels in a similar environment within the same tumor) exhibiting markedly different levels of vascular leakiness. Interstitial drug delivery is often unpredictable due to this structural feature.

Once a therapeutic agent has entered the tissue compartment, it must fight against other physiological factors. The third barrier is the actual journey therapeutic molecules must take to selectively target and eradicate cancer cells. This process is otherwise known as "interstitial transport". The ability of the tumor interstitial matrix to limit transport of a desired agent possessing single or multiple physiochemical features (such as charge, size and shape of molecule) is a significant problem.[41,43–45] These factors must be taken into account whenever possible. To date, cationic liposomes (positively charged drug carrier molecules) have been used to selectively deliver therapeutic molecules to target, e.g. the p53 and interferon-beta genes, and to deliver antisense oligonucleotides and other therapies to tumors.[46–48] An issue of particular concern is when therapies are delivered by the intravenous route of administration and the intended target is located within the tumor interstitial matrix. Studies have shown that cationic liposomes (approx. ~150 nm in size) preferentially target the intravascular compartment of tumors and are not as likely to penetrate the interstitial matrix as their similarly sized anionic and electroneutral counterparts.[49–51] The same issue holds true for micelles, nanoparticles

and other potential carriers of peptide and protein therapeutics of a similar size and charge.

The next barrier limits the extent to which drugs can penetrate the tumor interstitium and thus reach their intended cellular targets. Barrier #4 is elevated, interstitial fluid pressure (IFP) also known as *interstitial hypertension*. An increase in tumor IFP reduces the transcapillary pressure gradient and drives an outward pressure gradient (or fluid flux) over the capillary wall.[52] An IFP gradient from the tumor center to the periphery is responsible for the characteristic outward convection tissue gradient commonly linking elevated IFP with limited delivery and transport.[41,52] *In vivo* investigations involving the use of isolated tumor preparations revealed significant fluid in areas surrounding the tumor periphery as a result of elevated tumor pressures. In these studies, mammary carcinomas MTW9 and Walker 256 were transplanted in rats, and the net fluid loss was estimated at around 0.14 to 0.22 ml/hr per gram of tissue. A highly reproducible measure of increased hydrostatic pressure was the main reason for the significant loss of fluid.[53,54]

In areas near the tumor periphery bordering the interface of normal host tissues, the IFP was estimated at near 0 mm Hg.[40,55] Tumor interstitial pressure is, however, quite variable and region-dependent; IFP is closer to zero near the tumor periphery but near the center of the tumor, it is significantly higher and more uniform.[55] A "wick-in-needle" (WIN) technique *(see Ref. 56 for a full description of the technique)* was used to evaluate IFP in skin (melanoma) and cervical carcinomas and values were estimated around 45 and 36 mm Hg, respectively. In another study, the average IFP values ranged between 5.8 to 22.8 mm Hg.[52,56] A strong correlation exists between IFP and tumor size: the larger the tumor the higher IFP values in human and animal tumors.[56] Another report showed that the mean IFP for human breast and liver tumors derived from a primary colorectal tumor was estimated at around 33 and 21 mm Hg, respectively.[56,57] No matter the experimental tumor model used to investigate IFP, it is clearly evident that the interstitial fluid pressure is significantly higher in tumors compared with normal tissues and is associated with poor prognosis.

Due to significant vascular permeability and insufficient lymphatic drainage, the sum accumulation of fluid pressure in the vascular compartment (~aka microvascular pressure (MVP)) directly influences IFP.[58,59] MVP is dependent on differences in both arteriovenous pressure and

"geometeric and viscous resistance" to blood flow; experimental evidence supports higher geometric and viscous resistance in tumors, estimated to be 1–2 magnitudes higher than in healthy tissues.[41,56,57,60] To date, a mechanism-based understanding of elevated IFP is not completely mapped out. A combination of factors such as increased vessel permeability, dysfunctional lymph vessels and structural alternations in the basic design and composition of the interstitial matrix compared with normal tissues are likely contributing factors.

Clinical investigations support a relationship between IFP and patient survival that is seemingly independent of prognostic factors. Cervical cancer patients treated with radiotherapy with relatively high IFP tumors were more likely to present within the pelvis and at untreated anatomical locations, compared with those with tumors with lower IFP.[61] Moreover, disease-free survival for patients with low and relatively high IFP (>19 mm Hg) was 68% and 34%, respectively.[61] The relationship between IFP and angiogenesis was investigated in studies involving the use of intravital microscopy. Tumor IFP was found to be highly dependent on neovascularization. Immature tumors without a developed vascular supply (stage 1 of development) had reportedly lower IFP values compared with the same tumors at a more established stage of physiological development (stages 2 and 3).[55] One cannot rule out the possibility that other inactive tumor stage-dependent factors might turn on later during stages 2 and 3 of the tumor development, contributing at least in part to interstitial hypertension. Elevated IFP is thus an important factor impeding effective penetration and distribution of therapeutics, including peptides and proteins.

How efficiently peptide and protein therapeutics travel through leaky blood vessels to desired target locations is regulated by the extent to which these barriers influence the process. In order to improve delivery, we seek to understand how experimental agents affect the density and diameter of tumor vessels, volume surface area, and blood flow. Highly sophisticated *in vivo* imaging techniques now offer ways to investigate effects of therapeutics on the structure and function of tumor-associated blood vessels.[62,63] An intravascular compartment and a formidable interstitial matrix may represent common features of all solid tumors, but tumors are unique in that they all possess a different microvessel structure and organization.

Comprehensive perfusion rate studies revealed the existence of at least three separate tumor zones. Starting from the outermost region of the tumor

and working towards the central core, the periphery is the most vascularized region (zone 1) and is often the intended target of most peptide therapeutics. Zone 2 represents the semi-necrotic region and is characterized by relatively lower perfusion rates compared with zone 1. The poorly perfused, highly necrotic zone 3 is devoid of blood vessels (avascular).

Tumor cells within approximately 110 μm of the vasculature are viable, and necrosis due to prolonged hypoxia is observed in regions exceeding this critical limit of nutritional support.[64] In this way, geometric organization and location of neoplastic cells in relation to the blood supply is critical for tumor progression.[31,65] It would also stand to reason that an increase in necrotic tissue mass results in limited perfusion of oxygen, nutrients, and therapies to these tumor areas. Several lines of evidence suggest that hypoxic conditions give rise to the potent upregulation of VEGF (vascular endothelial growth factor). Upregulation results in higher expression levels in areas deprived of oxygen compared with more highly vascularized, oxygen-rich regions. In this regard, it is not difficult to understand why tumors are difficult to treat. The extent to which a particular agent can exert a desired therapeutic effect is determined by the extent to which the intended target zone is affected by treatment.

Three main parameters are generally used to define dynamics involved in the transport of all circulating therapeutics. These are blood flow rate, transport across the vascular wall, and transport within the interstitial matrix. The rate of blood flow is proportional to the drop in blood pressure across a vascular bed.[41,57] The drop in pressure is inversely proportional to "geometric and viscous resistance". Rate of blood flow, therefore, depends on these specialized features of blood vessels, including the number of blood vessels, patterns of their branching, vessel length, and diameter. The most effective approaches to date take one or more of these parameters into account.

Unlike normal tissues, tumor vessels do not respond as well to vasoactive agents (i.e. histamine, bradykinin, and serotonin) normally used to regulate blood flow resulting from injuries or inflammatory stimuli. How then do blood vessels become leaky? Tumors secrete a multifunctional cytokine called VPF/VEGF (vascular permeability factor/vascular endothelial growth factor).[66] VEGF induces rapid and reversible increases in extravasation of proteins (a function discovered by Dvorak's group with underlying mechanisms by Ferrara and colleagues).[66–68] Relatively wide

inter-endothelial spaces are created, thus allowing for extravasation of proteins. It is important to note that not all tumor vessels are leaky; therefore, tumors exhibit spatial heterogeneity. Upon intravenous administration of stealth liposomes, perivascular localization of liposomes in tumor tissue was observed. This further supports the notion of heterogeneous distribution of hyperpermeable regions along the length of a single vessel.[11,69]

VEGF can exert an effect on tumor vessels that is 50 000 times more potent than histamine, and a number of significant physiological effects can result from the influence of VEGF on vascular permeability.[70] Some changes include extravasation of plasma proteins in tissue and elevated levels of cytoplasmic calcium. Specific changes to endothelial cells are alterations in cell morphology, patterns of migration, and gene expression. All of these changes are essential to the functional development of tumors. Since the lining of tumor vessels possesses relatively high affinity VEGF receptors (VEGF1 and VEGF2), over-expression of VEGF in tumors has formed the basis of many rational peptide and protein therapies against cancer.[67,70]

3.2. *The interstitial matrix: MMPs, collagen, invasion and metastasis*

In order for a primary tumor to expand beyond its local environment, cancer cells must first detach and migrate to (and establish growth at) a secondary tumor site. A successful journey involves degrading the basement membrane and invading the surrounding region to gain access to the intravascular compartment for the purpose of traveling to a favorable distant location. This process has been described elsewhere as the "three-step theory of invasion".[71] The first phase describes cellular attachment to the interstitial matrix through interactions with extracellular glycoproteins (i.e. laminin and fibronectin). The second step involves local proteolysis resulting from the release of specific hydrolytic enzymes synthesized by cancer cells, or generated by host cells which have been instructed by cancer cells to synthesize them. The third step involves the actual migration (or locomotion) of cells into areas structurally rearranged by hydrolytic enzymes.[71]

The tumor interstitial matrix is composed of multiple proteins involved in intercellular communication and in interactions of cells with components of the interstitial matrix. Cadherins, integrins, laminin, fibronectin, and

matrix metalloproteinases (MMPs) are but a few of the proteins shown to play a role in the functional regulation of invasion and metastasis.[71–76] These specialized components of the interstitial matrix perform important roles in both health and disease. It is, however, beyond the scope of this chapter to discuss each in detail. Due to the steady increase in the number of publications relating MMP and collagen function to tumor invasion and metastasis, some attention will be focused on these components.

The field of cancer research has benefited enormously from investigations into the structural and functional relationships of MMPs and cancer. MMPs are the interstitial enzymes that degrade collagen, but require Ca^{++} and Zn^{++} to exert function.[77,78] Of note is the regulation of MMP synthesis and functional action in tissues by the specific local action of MMP-inhibitors, without which the functional activity of MMPs might go unregulated in host tissues.

MMPs are involved in physiological processes, including bone remodeling and embryogenesis, and in pathological conditions such as tissue destruction, arthritis, cancer and other diseases.[73,74] MMPs degrade collagen in pathological tissues. The turnover rate of interstitial collagen in normal tissues is relatively slow compared with that of tumors, with an estimated half-life in years. Two types of MMPs involved in metastasis and in the rapid breakdown of collagen in tumors are MMP-2 and MMP-9; digestion of collagen type IV and type V have been reported for each, respectively.[74] Type IV collagen is the main component of the basement membrane, whereas type V is found in areas located between the basement membrane and interstitial stroma.[37,77–79]

The role of MMP-2 in angiogenesis and cancer has been investigated.[76] In one study, MMP-2 knockout mice demonstrated a reduced response to B16-BL6 and Lewis lung carcinoma cells when implanted intradermally. MMP-2 deficient mice exhibited significantly lower tumor growth, demonstrating 39 and 24% reduced growth in comparison with MMP-2 competent mice, respectively.[76] Subsequent studies later linked MMP-9 activity with invasion of high grade gliomas, and to effective therapeutic action of Interferon β-1b.[80,81]

A fragment of collagen IV α3 chain generated by MMP-9 proteolysis (aka ~tumstatin) inhibited angiogenesis associated with tumor progression, without exerting effects on the physiology of normal tissues. This is possible due to the over-expression of β3 integrin in tumors compared with normal tissues, and because tumstatin requires β3 integrin to exert its

therapeutic effect.[82] Mice deficient in MMP-9 were shown to have lower circulating levels of tumstatin linking MMP-9 with integrin-mediated tumor suppression. The product of MMP-9 proteolysis was later demonstrated to inhibit invasive properties of metastatic melanoma *in vivo* by triggering an intracellular signaling cascade.[83] Altogether, these data support an endogenous role of MMP-9 in tumor biology, including regulation, invasion and metastasis.

A number of additional components of the interstitial matrix offer benefits in terms of tissue organization and structure, many of which limit optimal delivery of larger protein therapeutics to tumors due to unfavorable physicochemical characteristics. These are glycosaminoglycans, proteoglycans, and high collagen content in stroma; several studies have investigated their structural and functional significance in normal and tumor tissues.[12,15,25,43,84] It was recently shown (with the use of noninvasive techniques) that successful modification of the tumor interstitial matrix can result in improved diffusive transport. In this study, the pregnancy hormone relaxin was administered to HST26T containing SCID mice and a significant increase in diffusion coefficients of IgG and dextran was observed.[85] The effect of relaxin on diffusive properties of IgG and dextran is likely due to upregulated functional effects of MMPs.[86] Elevated levels of relaxin associated with tissue remodeling in breast cancer patients have since been discovered.[85] Important findings resulted from a study of the effects of extracellular matrix composition, structure, and distribution of molecules in tumors. Investigations confirmed that diffusion of small proteins was not affected by tumor location; however, tumor location was an important consideration for diffusion of significantly larger protein molecules of a similar chemical composition. The diffusional hindrance of larger molecules correlated with relatively high collagen type I and fibrillar collagen content in the diffusion limiting site.[43] The design of the interstitial matrix, including the role of various protein components, is thus critical in the regulation of cancer and progression of disease.

3.3. *Overcoming barriers: Exploiting tumor physiology for therapeutic gain*

Depending on tumor type and the tumor-associated microenvironment, optimizing delivery of peptides and protein therapeutics to tumors will require success with at least one of two different approaches. The first

approach is to circumvent one or more tumor physiological barriers. The second is to temporarily impair physiological barrier function(s) prior to administering interstitial targeting agents. The realization of completely eliminating or suppressing any one of these barriers is no easy task.

Tumor vascular targeting might represent a rational way to circumvent elevated tumor interstitial pressures and other impeding factors of the interstitial matrix. However, for tumors to respond optimally to treatment, sufficient access of therapeutics to the tumor blood supply is essential. How can this be accomplished? In one study, Hong *et al.*[87] successfully used adenosomes (adenovirus proteins combined with cationic liposomes) to deliver AAV/CMV-LacZ to human endothelial cells. Peptide-mediated therapy involving specific ligands has been used to deliver genes to endothelial cells. In one study, two different derivatized RGD peptides delivered cationic lipid-plasmid DNA to human umbilical derived endothelial cells (HEVEC), and a 4-fold increase in transfection efficiency was reported.[88] Several studies have reported successful efforts to deliver peptide-based therapeutics to endothelial cells *in vitro* and *in vivo*. By way of example, a lipid-mediated *peptide nucleic acid* (PNA) agent was used to deliver peptide therapeutics to pulmonary endothelial cells *in vivo*.[89] The peptide derived from the LDL receptor (LDLr) binding domain of apolipoprotein E (apo E) improved the uptake of liposomes by endothelial cells lining the brain. The authors note that non-protein coated liposomes were not successfully taken up by the brain endothelial cells.[90] Also noteworthy is the opportunity to target therapeutics to the brain while avoiding the highly elevated IFP normally associated with tumors in this anatomical location.

Natural and synthetic sources of angiostatic proteins and peptides have been evaluated and the majority of them demonstrate the ability to inhibit neovascularization *in vivo*.[35] Macromolecule-assisted delivery of these agents to tumor vessels could improve vascular recognition and outcomes associated with treatment.

Earlier in this chapter, the multicytokine function of VPF/VEGF in tumors was discussed. It is reasonable to discuss using VEGF to induce tumor vascular permeability to circulating therapies. The delivery of VEGF in sufficient levels to endothelia could result in venules that are more permeable to therapeutics. From this perspective both delivering VEGF directly to tumor vessels, and delivering plasmids encoding for VEGF to

this site as well, might represent equally effective approaches. To illustrate, when plasmid encoding for VEGF was injected as a VEGF-cationic liposome complex, gene expression was detected after 1 to 3 weeks, with DNA detected up to several months following the initial injection.[91] An *in vivo* quantitative measure of the effects of various growth factors (VEGF, PIGF-1 and PIGF-2 (platelet growth factors), and bFGF (basic fibroblast growth factor)) on the permeability of tumor vessels to circulating macromolecules (dextran, liposomes, and albumin) demonstrated that only VEGF significantly increased vascular permeability.[92] Such studies have clinical implications for tumor and non-tumor vascular related diseases.

In some situations a more practical approach could involve delivering therapeutic peptides or proteins directly to populations of neoplastic cells invading the tumor matrix. In this approach some specialized feature of neoplastic cells is usually exploited for therapeutic gain. These studies are usually investigated with the use of human tumor xenograft models in the presence of fully functioning physiological barriers; suboptimal to adequate levels of success is commonly associated with treatment outcome. Upon closer evaluation, better treatment outcomes would probably result if more clinically relevant therapeutic concentrations were delivered to the tumor interstitial compartment. Simply increasing the injected dose may improve delivery of therapeutics to the intended target over normal tissues, but the final dose should be optimized in relation to tumor and non-tumor targets to maximize therapeutic effect. The aim, however, is to improve drug location and duration of drug exposure at the intended site(s) of drug action; increasing the ratio of drug to tumor as opposed to normal tissue is a critical first step.

Over the last decade, several groups have investigated the use of agents to lower tumor IFP with the aim of reducing the pressure long enough to allow for better penetration of therapeutics into interstitial tumor areas. The eventual hope is to treat regions that would otherwise go unaffected by more conventional approaches. To summarize a few of these studies, the following agents have demonstrated the ability to lower tumor IFP: tumor necrosis factor-alpha (TNF-α); tumor necrosis factor-beta (Fc:TβRII); dexamethasone; pentoxifylline (PTX); and taxol.[93–97] In experimental animal models, several agents have been shown to lower tumor IFP.[93,94,96–103] Table 1 shows a list of 10 agents that have been shown to lower tumor interstitial fluid pressure, with a summary of the injected dose, route of

Table 1 Tumor models used to investigate effect of agents on tumor IFP and related properties. U87 (human glioblastoma); HSTS-26T (human soft tissue sarcoma); MCaIV (murine breast carcinoma); S-MEL (human melanoma); MeWo (human malignant melanoma derived from lymph node); KAT-4 (human thyroid carcinoma); LS174T (human colon adenocarcinoma); FSaII (fourth generation, fibrosarcoma); MATB-111 (rat mammary adenocarcinoma); NT [aka ~ CaNT] murine breast adenocarcinoma; C-IMC (chemically-induced mammary carcinoma).

The following agents have been shown to lower tumor interstitial fluid pressure					
Agent name	Injected dose	Injection route	Tumor type	Species/ strain	Result summary/Conclusions
					(Griffon-E et al., 1999)
(1) Taxol	40 mg/kg	tail vein	*U87mg *HSTS-26T *Mca-IV	Nude NCr/Sed C3H/Kam	*48 hrs post-injection*: ↑ tumor vascular diameter HSTS-26T & Mca-IV showed↓ IFP ($P < 0.05$) IFP of U87mg (taxol resistant) was not affected by taxol
					(Kristensen et al., 1996)
(2) TNF-α	500 μg/kg	tail vein	*S-MEL *P-MEL *MeWo	Male nude	*5 and 24 hrs post-injection:* All 3 tumors = ↓IFP by 50–70% ($P < 0.05$) " = ↓ MABP by 30% ($P < 0.01$) no pressure lowering effect observed at 24 hrs
					(Salinikov et al., 2005)
(3) Fc:TβRII	*Fc:TβRII;* 1 mg/kg *Dox;* 3 mg/kg, every 2 days for 2 wks	i.p.	*KAT-4	athymic C57 bl/6	Pretreatment with Fc:Tbeta RII ↑ doxorubicin efficacy FcTβRII normalized blood vessels FcTβRII and rh-IL-1 ↓ IFP