Sensitization of Cancer Cells for Chemo/Immuno/Radio-therapy

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Sensitization of Cancer Cells for Chemo/Immuno/Radio-therapy

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Preface

Cancer chemotherapy can be traced in the 1940s when mustine (the prototype nitrogen mustard anticancer chemotherapeutic drug) was injected into a patient with non-Hodgkin's lymphoma, resulting in a dramatic reduction in tumor masses [1]. Thereafter, we witnessed the discovery and the important application of several new drugs, such as methotrexate [2], 6-mercaptopurine (6-MP), vincristine (vinca alkaloid), and aminopterin (folic antagonists) [3]. Then the concept of combination chemotherapy was introduced in 1965 by James Holland, Emil Freireich, and Emil Frei, who administered methotrexate, vincristine, 6-mercaptopurine, and prednisone, together referred as the POMP regimen, which resulted in long-term remission in children with acute lymphoblastic leukemia (ALL). This combination approach was extended to the lymphomas by Vincent T. DeVita and George Canellos in the late 1960s, when it was found that nitrogen mustard, vincristine, procarbazine, and prednisone—known as the MOPP regimen—could cure patients with Hodgkin's and non-Hodgkin's lymphoma. Thereafter, new drugs were discovered, including taxanes, camptothecins, platinum-based agents, nitrosoureas, anthracyclines, and epipodophyllotoxins [4]. The successes of combination chemotherapy suggested that all cancers could be treated provided the correct combination of drugs at the correct doses and correct intervals were established. However, while chemotherapeutic drugs were effective with minimal knowledge of underlying mechanisms of action, new studies began to unravel the genetic nature of cancer and the development of targeted therapies.

Targeted therapies include monoclonal antibodies, cell-mediated immunotherapy, gene immunotherapy, and the development of inhibitors interfering with survival antiapoptotic signaling pathways in cancers. While these novel approaches have significantly improved the outcome of many cancer patients, there remains a major problem in the development of cancer resistance to conventional and novel cytotoxic therapies. Further, since most cytotoxic therapies mediate their activities by inducing programmed cell death, or apoptosis, tumor cells develop mechanisms to resist apoptosis and thus acquire a phenotype of cross-resistance to most cytotoxic stimuli. Therefore, there is an urgent need to unravel the underlying mechanisms of resistance at the biochemical and genetic levels and the development of agents that can reverse resistance, directly or in combination with other cytotoxics. The objective of this book is to select novel approaches developed to reverse tumor cell resistance to chemo/immuno/radio-therapy and the use of various sensitizing agents in combination with various cytotoxics [5]. This volume is by no means exhaustive of this subject matter, but primarily introduces several current approaches that have been developed by established investigators in the field. The volume is arbitrarily divided into several main topics, recognizing that the contents of several chapters in one topic can overlap with other topics.

There are several contributions on tumor cell sensitization based on approaches to target cell surface receptors and how such targeting agents sensitize tumor cells to apoptosis. Dr. Vollmers

and colleagues describe the use of monoclonal antibodies as sensitizing agents to reverse epithelial cancers to apoptosis. Dr. Penichet and colleagues developed monoclonal antibodies directed against the overexpressed transferrin receptor on tumor cells. They also genetically engineered a fusion protein that was found to be cytotoxic and also sensitizes tumor cells to various chemotherapeutic drugs. Dr. Bonavida and colleagues discuss the FDA-approved chimeric anti-CD20 mAb, rituximab, and its ability to sensitize drug-resistant B-NHL to apoptosis by various chemotherapeutic drugs. They describe rituximab-mediated inhibition of several anti-apoptotic and constitutively activated signaling pathways and that are responsible for chemosensitization. Dr. Sakai and colleagues examine the role of the TRAIL death receptor, DR5, and its upregulation by various agents, leading to sensitization of TRAIL-resistant tumor cells to TRAIL-induced apoptosis. It is noteworthy that TRAIL and agonist DR4/DR5 mAbs are currently being tested in phases I and II clinical trials for various cancers. Dr. Murphy and colleagues used proteasome inhibitors to sensitize tumor cells to immune-mediated apoptosis.

Several contributors describe their findings by targeting constitutively activated cell survival pathways in cancer. Dr. Kerbel and colleagues describe the use of anti-angiogenic inhibitors as chemosensitizing agents, with particular emphasis on metastatic disease. Dr. McCubrey and colleagues describe the constitutively activated cell survival pathways, namely, the Raf/MEK/ERK and PI3/AKT pathways, and the use of cell membrane–permeable small-molecular-weight inhibitors that target these pathways and can be used as chemosensitizing agents. Drs. Rosato and Grant describe the use of histone deacetylase inhibitors in combination with other agents for the reversal of tumor cell resistance. Dr. Sorokin describes the role of eicosanoids in the regulation of tumor cell resistance to apoptosis and the various means to target these lipids in order to reverse chemoresistance.

There are several contributions that investigate targeting of transcription factors as sensitizing agents. Dr. Chatterjee and colleagues examine the relationship between the transcriptional regulation of survival pathways and inhibition of these pathways, and shifting the balance to reverse resistance. They describe the roles of Raf kinase inhibitory protein (RKIP) as apoptotic and signal transducer and activator of transcription (STAT3) as antiapoptotic and describe the opposing effects of these two gene products. Dr. Gambari describes novel RNA-DNA–based strategies as chemosensitizing agents by targeting selected mRNAs with antisense oligonucleotides or small interfering RNAs (siRNA) or targeting transcription factors with decoy oligonucleotides. Drs. Maina and Domo examine the beneficial effect of combining inhibitors of *p53* as sensitizing agents when used in combination with conventional chemo- and radio-therapies to reverse resistance. Dr. Bonavida and colleagues examine the role of various inhibitors, such as nitric oxide (NO) donors, as sensitizing agents leading to inhibition of the transcription factors NF-κB and Yin Yang1 (YY1). Inhibition of these transcription factors upregulates death receptors (FAS, DR5) and sensitizes tumors cells to FAS ligand and TRAIL-induced apoptosis. Dr. Aggarwal and colleagues used several natural products that inhibit NF-κB and sensitize tumor cells to both chemotherapy and radiation.

Due to the fact that the apoptotic pathways are dysregulated in cancer, and primarily there is overexpression of antiapoptotic gene products or underexpression of apoptotic gene products, sensitizing agents that can regulate these gene products and interfere with apoptotic pathways may reverse resistance when used in combination with other cytotoxics. Several contributors used such approaches. Dr. Johnson examines the application of inhibitors of the Bcl-2 family as chemo- and radio-sensitizers. These studies were undertaken both in vitro and in vivo for their potential clinical application. Dr. Johnston and colleagues also used the strategy of interfering with the dysregulated apoptotic pathways in cancer and describe various means to interfere with antiapoptotic pathways by using, for example, antisense and siRNA as sensitizing agents. Dr. Li and colleagues discuss the use of peptides and peptide mimetics as sensitizing agents and their possible application in clinical trials as a new approach for cancer therapy. Dr. Mayo and colleagues discuss the utility of nonpeptide mimetics to sensitize tumor cells when used in combination with subtoxic doses of chemotherapy and radiation. Drs. Sarkar and Lee discuss the effects of

combining isoflavones and conventional therapeutics. Isoflavones and derivatives exert many effects on cancer cells, such as regulating several survival pathways and apoptotic pathways. Drs. Schwenzer and Förster discuss antisense oligonucleotides and siRNA applications in therapy and their use in ongoing clinical trials.

The approach of tailored customizing therapy for individual cancer patients requires a thorough understanding of the genetic makeup of the patient and its cancer and the pharmacogenetics of drugs. Drs. Efferth and Wink discuss the pharmacogenetic approach to compare monogenetic disease with a more complex disease such as cancer. These studies open the way to design personalized custom-tailored therapy. Also, Drs. Stivala and her colleagues discuss the importance of how genetic abnormalities may influence the response to treatment. They also discuss current strategies to integrate pharmacogenetics into the development of anticancer drugs.

Clearly, this volume represents a broad overview of the field of cancer sensitization and introduces several novel approaches that can be used to reverse cancer resistance through the application of a variety of sensitizing agents. Readers are also encouraged to read several reviews on related topics. As editor, I wish to thank all of the contributors, without whom this book could not have been realized. In addition, I acknowledge the administrative and technical support of Maggie Yang and Erica Keng for their diligent and professional input. Lastly, I wish to thank my wife and two sons for their unconditional support during the preparation of this volume.

Benjamin Bonavida, PhD

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COLOR PLATES

COLOR PLATE 1 Immunohistochemical staining of antibody PAM-1 on different precursor lesions of prostate carcinoma. Paraffin sections were stained with hematoxylin-eosin, unspecific human IgM as a negative control and antibody PAM-1: **A.** Normal prostate tissue and low grade prostate intraepithelial neoplasia (PIN). **B.** High-grade PIN. **D.** Prostate adenocarcinoma and high grade PIN (original magnification, ×100)

COLOR PLATE 2 SC-1 induced apoptosis in vitro and in vivo. Cleavage of cytokeratin 18 in SC-1-treated apoptotic stomach carcinoma cells in vitro. Immunohistochemical staining of cytospin preparations reveals that 24 hours after induction of apoptosis, cleavage of cytokeratin 18 starts **(B)**, and after 48 hours, apoptotic bodies are released from the cells **(C)**. In **(A)**, a nonapoptotic cell is shown. (Original magnification × 400) DNA fragmentation in SC-1-treated apoptotic stomach carcinoma cells in vivo. Apoptotic stomach carcinoma cells in a metastasised tumour of a 50-year-old patient after treatment with the antibody SC-1. The patient received a single dose of antibody SC-1 and the tumour specimen was investigated for SC-1 induced apoptosis using the Klenow FragEL DNA fragmentation Kit (Oncogene, Boston). **D, G.** Control antibody CK8, tumour cells are stained. **E, H.** Positive control, all cell nuclei are stained. **F, I.** only the nuclei of apoptotic tumor cells are stained and normal not malignant tissue is not affected. (Original magnification, ×100 ***(D, E, F)***/×200 ***(G, H, I)***)

COLOR PLATE 3 SAM-6 induced apoptosis: mode of action. Immunofluorescence of SAM-6 endocytosis. Pancreas carcinoma cells BXPC-3 were incubate with fluorochrome labeled SAM-6 antibody. After 30, 60, and 90 minutes cells were exposed on slides, fixed, and analyzed using confocal microscopy. 30 minutes, antibody binding; 60 minutes, "capping"; 90 minute antibody SAM-6 is completely internalized into the cell. Sudan III staining of neutral lipids in SAM-6 treated tumor cells. Pancreas carcinoma cells BXPC-3 were incubated with antibody SAM-6 antibody or for 2, 24, and 48 hours. An accumulation of red stained lipid droplets is visible in antibody SAM-6 treated tumor cells. Magnification ×200. Scanning electron microscopy of SAM-6 antibody-induced apoptosis. Stomach carcinoma cells 23132/87 were incubated with antibody SAM-6 for 2, 24 and 48 hours. Samples were proceeded for scanning electron microscopy and analyzed by ZEISS DSM 962. On the SAM-6 treated tumor cells apoptotic effects such as stress fibers, loss of cell-cell contacts, and clusters of apoptotic bodies are visible

COLOR PLATE 4 In vivo treatment of Her-2 positive MDA231-H2N human mammary tumors grown orthotopically in female SCID mice. **A.** Antitumor effects of low-dose metronomic cyclophosphamide (Ld CTX), maximum tolerated dose cyclophosphamide (MTD CTX), or trastuzumab alone, and combination regimens using low dose metronomic cyclophosphamide plus trastuzumab, or trastuzumab in combination with MTD cyclophosphamide. Arrows indicate time of MTD CTX dosing. **B.** Addition of second line therapies to tumors of MDA231-H2N

that were starting to fail (as shown in top panel **[A]** after around 100 days) Ld CTX plus trastuzumab therapies. Second-line regimens were as indicated, with the addition of bevacizumab causing a further growth delay of approximately 4 weeks. (Adapted from du Manoir JM, Francia G, Man S, et al. Strategies for delaying or treating in vivo acquired resistance to trastuzumab in human breast cancer xenografts. Clin Cancer Res 2006, 12:904–916.

COLOR PLATE 5 An example of how chronic combination oral metronomic low-dose CTX and UFT prolongs survival of mice with advanced metastatic disease. (From Munoz R, Man S, Shaked Y, et al. Highly efficacious nontoxic preclinical treatment for advanced metastatic breast cancer using combination oral UFT-cyclophosphamide metronomic chemotherapy. Cancer Res 2006, 66:3386–3391.) **A.** 231/LM2-4 human breast metastatic variant cells were orthotopically injected into the MFPs of 6- to 8-week-old CB17 SCID mice. When tumors reached volumes of approximately 200 mm^3, treatment with either vehicle control, or 15 mg/kg/day UFT by gavage, or 20 mg/kg per day CTX through the drinking water, or a combination of CTX and UFT treatments was initiated. Tumors were measured weekly and tumor volume was plotted accordingly. Arrow indicates time of initiation of treatment. **B.** 6-week-old CB-17 SCID mice were recipients of 231/LM2-4 transplanted cells. When tumors reached 400 mm^3 (which took approximately 3 weeks) primary tumors were surgically removed. Treatment with vehicle control, 15 mg/kg per day UFT by gavage, 20 mg/kg per day CTX through the drinking water, or the daily combination of metronomic UFT and CTX, were initiated 3 weeks after surgery on a daily non-stop basis. For example, in the experiment shown in **(B),** the duration of the therapy was 140 days, and was initiated on day 43, 3 weeks after surgery, with termination at day 183. Mice were monitored frequently according to the institutional guidelines. A Kaplan-Meier survival curve was plotted accordingly for all treated groups, as indicated in the figure. **A, B.** n = 7–9/group. NS = normal saline, Veh = vehicle control (0.1% HPMC). Note that effects on primary tumor **(A)** were minor and in no way predictive of the survival benefits seen with UFT and CTX on metastatic disease (recorded as survival)

COLOR PLATE 6 Metabolism of arachidonic acid and synthesis of major eicosanoids. Reactions catalyzed by cyclooxygenases are shown in the pink field, reactions catalyzed by lipoxygenases are shown in the green field and reactions catalyzed by CYP monooxidases are shown in the yellow field. Also shown are five major prostanoids, which are synthesized by prostaglandin synthases from PGH_2, and LTA_4 converted from product of lipoxygenase reaction, which is further converted to LTB_4 by LTA_4 hydrolase

COLOR PLATE 7 Structure of PNA-DNA-PNA chimeras targeting NF-kappaB–related proteins. The molecular structures and the sequence of the double-stranded PNA-DNA-PNA chimera mimicking NF-kappaB binding sites are modified from Borgatti et al. [116], Romanelli et al. [118], and Gambari et al. [109]

COLOR PLATE 8 Mechanism of tumor cell sensitization to Fas-L–induced apoptosis by IFN-γ: Pivotal role of NO. IFN-γ or other agents, such as TNF-a, IL-1, or LPS, upregulate NF-kB, which in turn regulates positively the transcription of NOSII. NOSII catalyses the biosynthesis of NO by L-arginine. NO can also be released in the cytosol by treatment of cells with an NO donor such as SNAP or DETANONOate. Free nitric oxide may react with O_2 (discontinuous line), resulting in the formation of reactive nitrogen species (RNS) such as ($ONOO^-$), which upregulate Fas and cause oxidative damage in protein and nucleic acids leading to apoptosis. Alternatively (continuous line), NO or NO^+ ion is capable of forming S-nitrosothiols resulting in S-nitrosylation of several proteins, including YY1, which acts as a repressor of Fas transcription. Thus, inducible levels of Fas by NO are able to overcome tumor resistance to Fas-L and sensitize them to Fas-L–mediated apoptosis

COLOR PLATE 9 Mechanism of tumor cell sensitization to TRAIL-induced apoptosis by NO. Treatment of several tumor cell lines with NO donors such as DETANONOate and TRAIL results in apoptosis and synergy is achieved. The synergy is the result of complementation in which each agent partially activates the apoptotic pathway and the combination results in apoptosis. The signal provided by NO partially inhibits NF-kB activity, and this leads to downregulation of antiapoptotic proteins of the Bcl-2 family such as Bcl-xL, and inhibition of cIAP family members (i.e.,

XIAP, cIAP-1, cIAP-2). In addition DETANONOate also partially activates the mitochondria and release of modest amounts of cytochrome C and Smac/DIABLO into the cytosol in the absence of caspase-9 activation. The NO-induced NF-kB suppression also inhibits the negative transcriptional regulator of DR5, YY1, resulting in DR5 upregulation. Thus, the combination treatment with TRAIL and DETANONOate results in significant activation of the mitochondria and release of high levels of cytochrome C and Smac/DIABLO, activation of caspases-9 and -3, promoting apoptosis. The role of Bcl-xL in the regulation of TRAIL apoptosis has been corroborated by the use of the chemical inhibitor 2MAM-A3 in several cell lines, which also sensitized the cells to apoptosis

COLOR PLATE 10 Model of the G6PD Aachen tetramer. This G6PD variant has originally been described by Kahn et al. [253]. The mutation in the G6PD Aachen variant has been determined by us previously [254]. A mutation 1089C>G results in a predicted amino acid change 363Asn>Lys. The 1089C>G point mutation is unique, but produces the identical amino acid change found in another variant of G6PD deficiency, G6PD Loma Linda. The 363Asn>Lys exchange in G6PD Loma Linda is caused by a 1089C>A mutation [255]. Using the available three-dimensional structure of the human G6PD tetrameric protein complex [256], the location of the point mutation of amino acid 363 in G6PD Aachen is found at the surface of a monomer in close proximity to $NADP^+$ and more than 20Å away from the glucose-6-phosphate binding site. This residue is probably involved in $NADP^+$ binding that in turn is required for tetramer stability [256]. Thus, Arg363 may be required to indirectly maintain the structural integrity of the functional unit. Replacing it with a positively charged Lys residue would lead to charge–charge repulsion between Lys363 and $NADP^+$, thus affecting $NADP^+$ binding and tetramer formation. The two pairs of dimer-forming monomers are colored in ice blue/blue and in orange/red. The mutation site (Asn363) and the cofactor $NADP^+$ are shown in van der Waals representation in purple and grey, respectively. The conserved eight-residue peptide RIDHYLGK corresponding to the substrate binding site is colored in yellow. The figure was prepared from the crystal structure 1QKI using the program VMD [257]

COLOR PLATE 11 Molecular modeling of SNP-based variants of MGMT. **A.** Wild-type structure of MGMT. Mutation sites are shown in van der Waals representation. Color codes of helices: red, N-terminal α-helices, blue, DNA recognition site with helix-turn-helix motif. The second DNA recognition site binds to the major groove of DNA, yellow, 3–10 helix with conserved Pro-Cys-His-Arg sequence. The active Cys145 is located here. Color code of loops: orange, Asn-hinge that joins the DNA recognition helix and the active site. It also provides 40% of the contact between N-terminal and C-terminal domains, white, DNA binding wing. O6-alkylguanine lies between the binding wing and the recognition helix. Color code of side chains shown in CPK representation: per atom, conserved active Pro-Cys-Arg sequence, pink, Zn^{2+} binding site (residues C5, C24, H29, H85). Cys5 is missing, since it is not resolved in the crystal structure 1QNT. **B.** Localization of amino acid change and overlap of structures of all mutants. Mutated side chains are shown in van der Waals representations. (Reprinted from Schwarzl SM, Smith JC, Kaina B, et al. Molecular modeling of O6-methylguanine-DNA methyltransferase mutant proteins encoded by single nucleotide polymorphisms. Int J Mol Med 2005, 16:553–557.)

Part I
Sensitization via Membrane-Bound Receptors

Chapter 1
Sensitization of Epithelial Cancer Cells with Human Monoclonal Antibodies

H. Peter Vollmers and Stephanie Brändlein

1 Natural IgM Antibodies

Natural IgM antibodies are part of the innate immunity and important components of first line defense mechanisms [1–3]. They are germ-line encoded, and are produced by a small subset of B lymphocytes, B1, or CD5+ cells [4–6]. The repertoire and reactivity pattern of natural antibodies is remarkably stable within each species and even between species. This genetic stability seems to be the result of an evolutionary selection process providing an inherent legacy of specificities capable of protecting against pathogen invasion, malignant cells, and other harmful alterations. Natural IgM antibodies have been shown to be involved in early recognition of external invaders such as bacteria and viruses [3, 7], but these natural IgM antibodies also seem to be involved in recognition and elimination of precancerous and cancerous lesions [8–12].

Although coded by a limited set of germ-line immunoglobulin genes, these IgM antibodies give a sufficient protection without additional mutational adaptation [13–18]. This is only possible, if IgM antibodies do not detect single structures, but instead bind to patterns of conservative structures, which are expressed by "non-self" structures. Furthermore, this recognition system guarantees that the innate immune response need not follow all mutational changes. However, natural antibodies are ideal antitumor weapons, because even if they have low affinity and show some oligo-reactivity, they have some unique properties that antibodies produced by xeno-immunization or phage display-technique do not have: They are tumor specific by nature.

In an extensive investigation of the antitumor defense in humans, several tumor-specific IgM antibodies could be isolated [8, 19, 20]. All these human monoclonal antibodies analyzed so far have some typical features in common. They are pentameric molecules, coded by specific germ-line families and they are equipped mainly with lambda chains, in contrast to the majority of circulating antibodies [8]. These natural IgM antibodies preferentially bind to tumor-specific carbohydrate epitopes on post-transcriptionally modified cell surface antigens [8, 21, 22]. Another typical feature of the natural IgM antibodies is their ability to induce apoptosis in malignant cells in a death domain independent way [8, 12, 21, 23–26]. This is done for example by blocking of growth-factor receptors, cross-linking of modified anticomplement receptors or by increasing the intracellular level of neutral lipids [12, 21, 22, 26] (Fig. 1.1).

2 Growth Factor Receptors

Cancer cells are hyper-proliferating cells, with a huge need of growth factors [27, 28]. Epidermal growth factor receptors (EGF-Rs) are therefore often found overexpressed on a variety of malignant cells [29]. Antibodies binding to EGFRs have been used to block these receptors and to inhibit the binding of the specific growth factor ligands [30]. The effect of these antibodies can be

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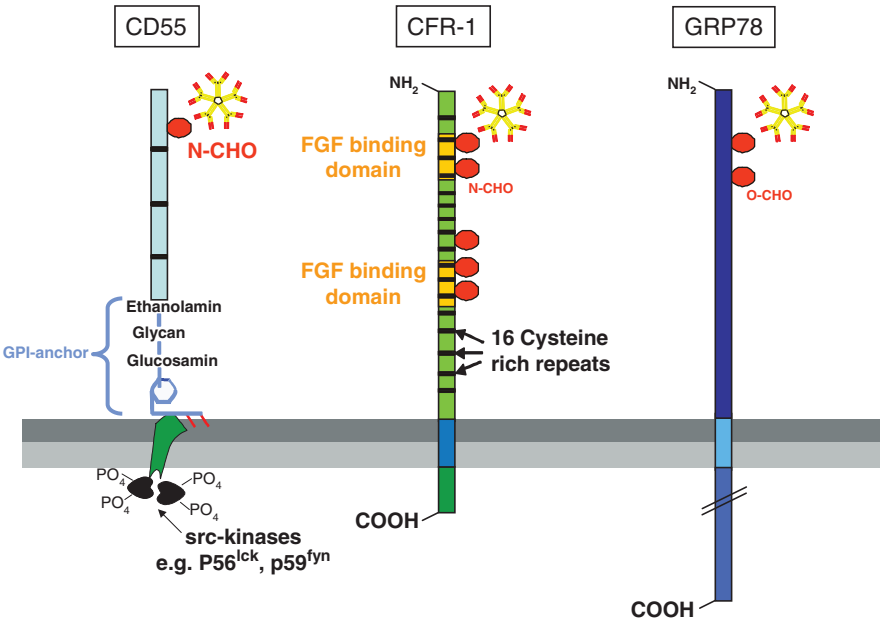

FIGURE 1.1. Tumor-specific post-transcriptionally modified cell surface antigens detected by natural IgM antibodies

enhanced in combinatorial approaches with conventional methods like radiation and chemotherapy and vice versa [31–35]. In breast cancer trials the monotherapeutic effect of trastuzumab (Herceptin) could clearly be enhanced in a synergistic manner when the antibody was used in combination with chemotherapy [32, 36–39]. Similar results were obtained for panitumumab (ABX-EGF) and cetuximab (Erbitux) in numerous cancer models, including lung, kidney, and colon [40].

The natural monoclonal IgM antibody PAM-1 was isolated from a patient with a stomach carcinoma and binds to a tumor-specific isoform of CFR-1 (Cysteine-rich fibroblastic growth factor receptor) [9, 22]. The CFR-1 is a 130-kDa integral membrane glycoprotein, homologous to CFR-1 (cysteine-rich fibroblast growth factor receptor), which has so far only been detected and described in Golgi of embryonic chicken cells and CHO cells [41]. The receptor is homologous to a rat protein, cloned as a Golgi-specific protein, designated MG160, which is involved in the processing and secretion of growth factors and was recently found in pancreatic cancer [42–46]. The human homologue, E-selectin ligand 1 (ESL-1), is a cytokine, expressed on myeloid and some lymphoma cells and is modulated by cell adhesion molecules that cause the binding of neutrophils to the endothelium [47, 48].

PAM-1 binds to a N-linked carbohydrate epitope which is specific for a post-transcriptionally modified isoform of CFR-1 (see Fig. 1.1) [22]. CFR-1/PAM-1 is expressed on almost all epithelial cancers of every type and origin, but not on healthy tissue [9, 22]. It is also found on precursor lesions such as *H. pylori*–induced gastritis, intestinal metaplasia and dysplasia of the stomach, ulcerative colitis–related dysplasia and adenomas of the colon, Barrett metaplasia and dysplasia of the esophagus, squamous cell metaplasia and dysplasia of the lung, cervical intraepithelial neoplasia I–III, ductal and lobular carcinoma in situ of the breast and prostate intraepithelial neoplasia (Fig. 1.2) [9, 10, 22]. PAM-1 inhibits tumor growth in vitro and in animal systems, by inducing apoptosis [25]. The effect is not dependent on the pentameric form and cross-linking, suggesting a similar mechanism as described for anti-EGFR antibodies [30].

3 Complement Decay Molecules

Decay acceleration factors (DAF) are surface receptors that protect host tissues from complement activation. They prevent cell damage by dissociating the classical and alternative pathway C3 convertases [49, 50]. CD55/DAF is such a complement

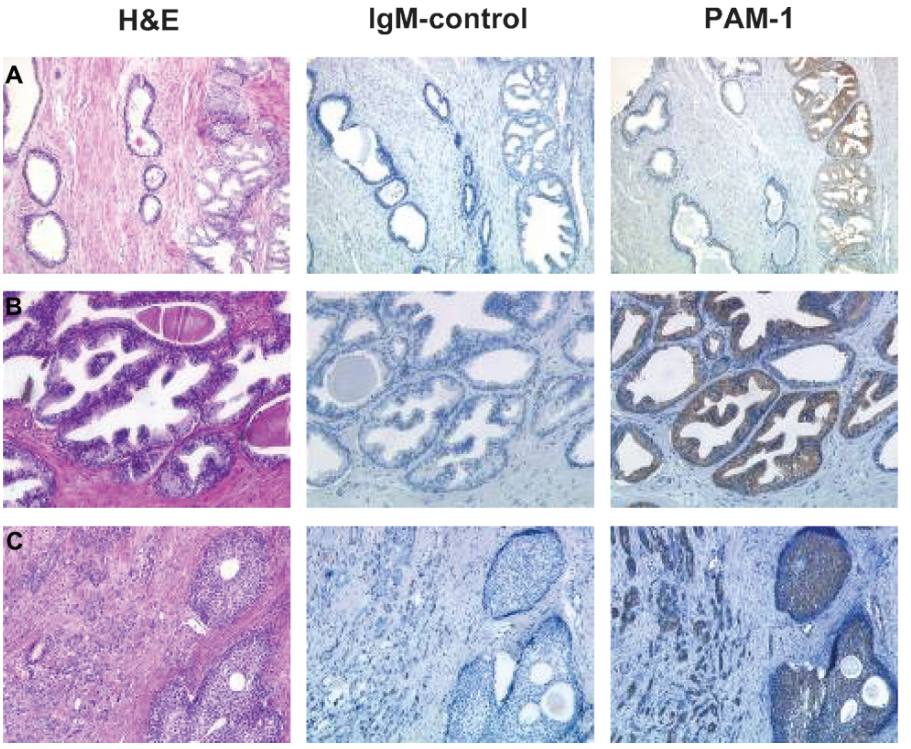

FIGURE 1.2. Immunohistochemical staining of antibody PAM-1 on different precursor lesions of prostate carcinoma. Paraffin sections were stained with hematoxylin-eosin, unspecific human IgM as a negative control and antibody PAM-1: **A.** Normal prostate tissue and low grade prostate intraepithelial neoplasia (PIN). **B.** High-grade PIN. **C.** Prostate adenocarcinoma and high grade PIN (original magnification, ×100) (*See Color Plates*)

regulatory protein, which is expressed in two different isoforms generated by differential splicing. While DAF-A is secreted from cells, DAF-B is linked to cells by a glycosylphosphatidylinositol (GPI-) anchor [51]. Both forms are further modified by different glycosylation patterns, resulting in sizes of 55–100 kDa molecular weight [52]. DAF-B is expressed on all cell types that can get in contact with the complement system [53].

Malignant cells often over-express DAF/CD55 molecules, to increase their protection level against complement attacks [49, 54]. Over-expression was found in, e.g., breast, colon, and stomach carcinoma [55–57]. This over-expression makes DAF a suitable target for cancer vaccines in the treatment of colon carcinomas [58]. Vaccination with a human anti-idiotype antibody that mimics DAF was used as an adjuvant treatment of colon carcinoma and resulted in an activation of a cellular antitumor response [59].

On the other hand, this over-expression of DAF and other complement inactivating molecules limits therapeutical approaches which depend on the help of complement, like in antibody-dependent cellular cytotoxicity. However, if malignant cells over-express and/or modify protection molecules with cell-specific alterations, these new epitopes can be used as targets for therapeutical approaches. The human monoclonal antibody SC-1, which was isolated from a patient with a signet ring cell carcinoma of the stomach [24], reacts with a N-linked carbohydrate epitope present on an isoform of DAF-B (subsequently named $DAF^{SC\text{-}1}$) with a molecular weight of approximately 82 kDa (see Fig. 1.1) [21]. The antibody reacts with over 70% of all diffuse-type and intestinal-type gastric adenocarcinomas [60]. Clinical studies have shown that specific induction of regression and apoptosis can be induced in primary stomach cancers without any detected toxic cross-reactivity to normal tissue [61].

Binding of SC-1 induces specific apoptosis of stomach carcinoma cells both in vitro and in

experimental in vivo systems (Fig. 1.3) [23, 62]. The effect depends on the cross-linking activity of the antibody which most likely inactivates the complement decay molecules. Shortly after binding, caspase-3 and -8 are activated resulting in cleavage of cytokeratin 18. Furthermore, there is a down-regulation of topoisomerase II, a short increase in the intracellular Ca^{2+}-concentration, which is not necessary for the apoptotic event but seems to be in involved in the regulation of DAF^{SC-1} expression [21, 62].

4 Heat Shock Proteins

Stress or heat shock proteins (HSPs) are ubiquitous and highly conserved cytoprotective proteins [63]. They play an essential role in intracellular

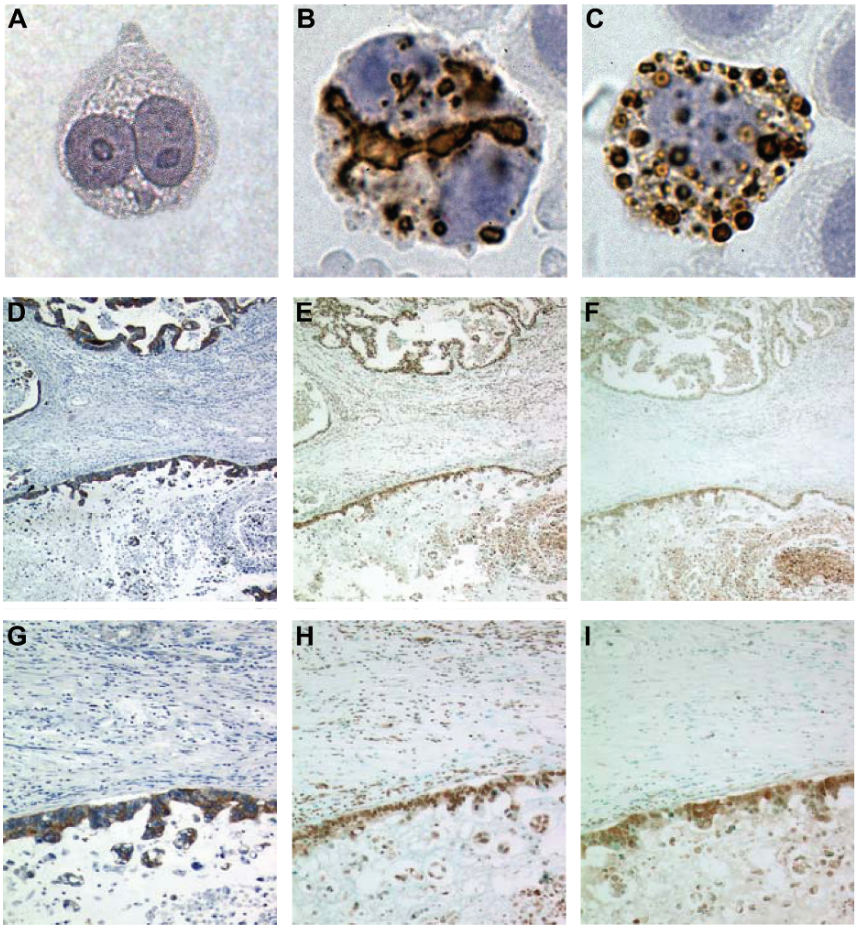

FIGURE 1.3. SC-1 induced apoptosis in vitro and in vivo. Cleavage of cytokeratin 18 in SC-1-treated apoptotic stomach carcinoma cells in vitro. Immunohistochemical staining of cytospin preparations reveals that 24 hours after induction of apoptosis, cleavage of cytokeratin 18 starts **(B)**, and after 48 hours, apoptotic bodies are released from the cells **(C)**. In **(A)**, a nonapoptotic cell is shown. (Original magnification × 400) DNA fragmentation in SC-1-treated apoptotic stomach carcinoma cells in vivo. Apoptotic stomach carcinoma cells in a metastasised tumour of a 50-year-old patient after treatment with the antibody SC-1. The patient received a single dose of antibody SC-1 and the tumour specimen was investigated for SC-1 induced apoptosis using the Klenow FragEL DNA fragmentation Kit (Oncogene, Boston). **D, G.** Control antibody CK8, tumour cells are stained. **E, H.** Positive control, all cell nuclei are stained. **F, I.** only the nuclei of apoptotic tumor cells are stained and normal not malignant tissue is not affected. (Original magnification, ×100 ***(D, E, F)***/×200 ***(G, H, I)***) (*See Color Plates*)

"housekeeping" by assisting the correct folding of nascent and stress-accumulated misfolded proteins and preventing their aggregation [64, 65]. HSPs allow the cells to survive to otherwise lethal conditions and play an essential role in tumor growth. They promote autonomous cell proliferation and inhibit death pathways induced by therapeutical approaches. Their expression in malignant cells is closely associated with a poor prognosis and resistance to therapy [66].

GRP78, also referred to as BiP, is a member of the HSP70 family [67]. It is induced in a wide variety of cancer cells and cancer biopsy tissues [68], and contributes to tumor growth and drug resistance [69].

However, the discovery of GRP78 expression on the cell surface of cancer cells further leads to the development of new therapeutic approaches targeted against cancer [70]. The human monoclonal antibody SAM-6 was isolated from a gastric cancer patient [26]. In binding and functional studies it was found that the SAM-6. reactivity is restricted to malignant tissue [26, 71]. The binding of SAM-6 could be removed by glycosidase treatment of the target cell, indicating a carbohydrate epitope of the antibody [72]. This expression of post-transcriptionally modified carbo-epitopes seems to be a common feature for malignant cells and was already proven for a series of other tumor-specific human monoclonal IgM antibodies [8, 21, 22].

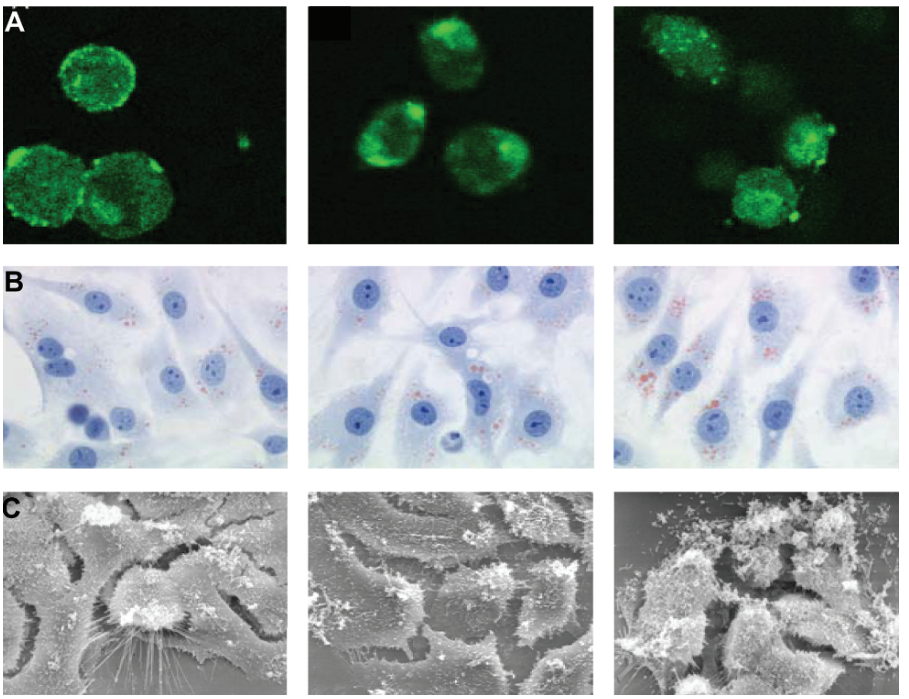

FIGURE 1.4. SAM-6 induced apoptosis: mode of action. Immunofluorescence of SAM-6 endocytosis. Pancreas carcinoma cells BXPC-3 were incubate with fluorochrome labeled SAM-6 antibody. After 30, 60, and 90 minutes cells were exposed on slides, fixed, and analyzed using confocal microscopy. 30 minutes, antibody binding; 60 minutes, "capping"; 90 minute antibody SAM-6 is completely internalized into the cell. Sudan III staining of neutral lipids in SAM-6 treated tumor cells. Pancreas carcinoma cells BXPC-3 were incubated with antibody SAM-6 antibody or for 2, 24, and 48 hours. An accumulation of red stained lipid droplets is visible in antibody SAM-6 treated tumor cells. Magnification ×200. Scanning electron microscopy of SAM-6 antibody-induced apoptosis. Stomach carcinoma cells 23132/87 were incubated with antibody SAM-6 for 2, 24 and 48 hours. Samples were proceeded for scanning electron microscopy and analyzed by ZEISS DSM 962. On the SAM-6 treated tumor cells apoptotic effects such as stress fibers, loss of cell-cell contacts, and clusters of apoptotic bodies are visible (*See Color Plates*)

The SAM-6 receptor is a tumor-specific isoform of GRP78, the epitope an O-linked carbohydrate (see Fig. 1.1) [72].

The antibody SAM-6 also binds to oxLDL and induces an excess of intracellular lipids, by overfeeding malignant cells with oxLDL via a receptor-mediated endocytosis (Fig. 1.4) [71]. The treated cells over-accumulate depots of cholesterol and triglyceride esters. Lipids are essential for normal and malignant cells during growth and differentiation. The turnover is strictly regulated because an uncontrolled uptake and accumulation is cytotoxic and can lead to lipo-apoptosis, lipoptosis [26]. This was shown in several animal studies and was also described for some inherited and acquired human diseases [73, 74]. When lipids over-accumulate in non-adipose tissue due to over-nutrition, fatty acids enter deleterious pathways such as ceramide production, and can cause apoptosis [73]. In mice and rats it was shown that lipotoxic cardiomyopathy is caused by accumulation of cardiotoxic lipids, which can induce the death of cardiac monocytes [75, 76]. Similar data on heart failure induced by lipid accumulation were obtained for humans by analyzing post mortem samples [77, 78].

The lipid over-accumulation induced by antibody SAM-6 is tumor-specific, nonmalignant cells neither bind the antibody nor harvest lipids after incubation with it. Shortly after internalization of the antibody-oxLDL–receptor complex and formation of lipid depots cytochrome c is released by mitochondria. Followed by this initiator-caspases 8 and 9 and effector-caspases 3 and 6 are activated and the apoptotic cascade starts (see Fig. 1.4) [72]. The interference with the lipid content in tumor cells by antibodies might be a novel avenue of cancer therapy.

5 Summary

Cancer cells respond like nontransformed cells to apoptotic signals, but they normally have a higher level of resistance. They use specific internal and external molecular changes, which make them less sensitive to death signals. On the other hand, malignant cells very often use their aberrant glycosylation machinery to modify carbohydrate residues on surface receptors. Antibodies that bind to this cancer-specific epitopes can be used to make cancer cells more sensitive to conventional therapeutical approaches.

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Chapter 2
Targeting the Transferrin Receptor to Overcome Resistance to Anti-Cancer Agents

Tracy R. Daniels, Isabel I. Neacato, Gustavo Helguera, and Manuel L. Penichet

1 The Transferrin Receptor

The transferrin receptor (TfR) plays an important role in iron uptake and delivery [1]. The primary role of the TfR is to internalize iron through the binding of its natural ligand, the transferrin (Tf) protein that carries iron through the circulation. Iron is necessary for various cell processes such as respiration, metabolism, DNA synthesis and the proper functioning of various heme and nonheme proteins that require iron as a cofactor [1]. In addition, the TfR seems to be important for other processes such as cell growth and proliferation [1].

The TfR, a 180-kDa homodimeric glycoprotein, is a type II transmembrane receptor that has three important domains for its function (Fig. 2.1). It is composed of a C-terminal domain, also known as the ectodomain, a transmembrane region, and an N-terminal domain that is on the cytosolic side of the membrane. The ectodomain is important for binding to Tf for the internalization of iron. Two TfR genes have been identified, TfR1 and TfR2. Furthermore, the TfR2 gene produces two transcripts, α and β, that are produced by alternative splicing. TfR2α shows similarity with TfR1 in that they exhibit a 45% similarity and 66% homology in their ectodomain. However, the cytoplasmic domains of the two proteins demonstrate no similarity [1]. The TfR2β transcript lacks the transmembrane and cytoplasmic domains and its function remains unknown. TfR1 and TfR2α differ in cell surface expression and gene regulation. The TfR1 is ubiquitously expressed on normal cells at low levels. Increased TfR1 expression is observed on cells with a high proliferation rate, including cancer cells. TfR2 expression is limited to hepatocytes and enterocytes in the small intestine [1]. TfR2 expression has been found in some human cell lines such as B and myeloid cell lines as well as some cell lines derived from solid tumors [1]. TfR1 is post-transcriptionally regulated directly by intracellular iron levels as compared with TfR2 that is not. TfR2 is thought to be primarily regulated by the cell cycle and iron-bound Tf [1]. Thus both receptors differ considerably in expression and regulation indicating different roles in iron delivery. In addition, TfR1 has a 25-fold greater affinity for Tf relative to TfR2, indicating the main role of TfR1 in iron homeostasis [1].

Tf is an 80 kDa monomeric glycoprotein composed of two lobes; an N and C lobe that are separated by a short spacer sequence (reviewed in [1]). Each lobe is capable of binding one iron molecule. The number of iron molecules bound to Tf has an important effect on the affinity of Tf for the TfR. At physiological conditions, holo Tf or diferric Tf (two iron) has the greatest affinity followed by monoferric (one iron), while apo-Tf (no iron) has the lowest affinity for the receptor [1]. Thus, iron uptake by the cell is mediated mostly through the

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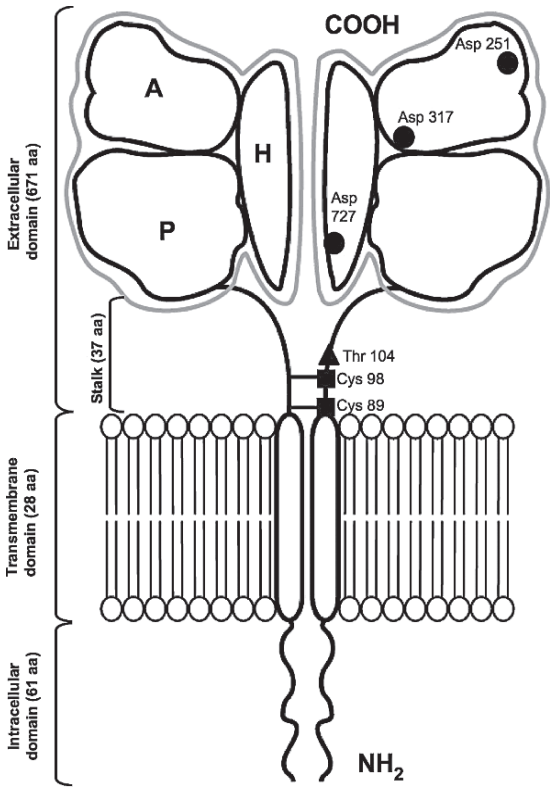

FIGURE 2.1. Schematic representation of the TfR. This receptor is a type II receptor found on the cell surface as a homodimer consisting of two monomers linked by disulfide bridges at cysteines 89 and 98 (■). The TfR contains an intracellular domain, a transmembrane domain, and a large extracellular domain. There is an O-linked glycosylation site at threonine 104 (▲) and three N-linked glycosylation sites on asparagine residues 251, 317, and 727 (●). The extracellular domain of the TfR consists of three subdomains: apical (A), helical (H) and protease-like domain (P). (Daniels et al. *Clinical Immunology* 2006, (121):144–158. Copyright 2006 Reprinted with the kind permission of Elsevier USA.)

interaction of diferric Tf and the TfR. The formation of a complex between diferric Tf and the TfR allows iron internalization into cells through a receptor-mediated endocytosis pathway (Fig. 2.2). This complex is internalized in a clathrin-coated pit into the cell and delivered into endosomes. Protons are pumped into the endosome causing an acidic change in the pH environment. This causes a conformational change in Tf that results in the release of iron. Iron can then be transported out of the endosome into the cytosol through a divalent metal transporter (DMT1). The Tf/TfR complex remains inside the endosome until it is brought to the cell surface where apo-Tf dissociates from the TfR and is then free to circulate and bind free iron.

Many studies have used the TfR as a target for the delivery of various therapeutic agents (reviewed in [2]). The high expression of the TfR in cancer cells (that can be 10 to 100-fold greater than normal cells), its cell surface accessibility, and constitutive recycling pathway make this receptor an attractive target for immunotherapy. Importantly, either Tf or anti-TfR antibodies can mediate delivery of molecules by TfR targeting. The following is a discussion of the various strategies that have utilized targeting of the TfR (summarized in Fig. 2.3 and Table 2.1) to overcome cancer cell resistance to therapy or to provide the first hit in the "two hit signal" model to sensitize resistant cells to chemotherapeutic agents as combination treatment strategies. Both strategies are of great importance in treating patients whose cancers have developed resistance to common therapies and have thus developed more aggressive malignancies.

2 Tf Conjugates to Overcome Chemoresistance

Doxorubicin (Adriamycin®) (ADR) is an anthracycline chemotherapeutic drug used to treat a variety of cancers. ADR blocks DNA synthesis along with the activity of topoisomerase II, an enzyme that helps to relax the coil and extend the DNA molecule prior to DNA synthesis or RNA transcription. When used as a single treatment modality, ADR often exhibits devastating side effects including cardiotoxicity, myelosuppression, nephrotoxicity, and extravasation [3]. Systemic drug toxicity is often attributed to quick diffusion throughout the body resulting in a homogeneous tissue distribution [4]. The potential benefits of ADR treatment may also be blocked by the development of drug resistant cancer cells. ADR resistance can be attributed to many molecular events. Includes the overexpression of the multi-drug resistance (MRP) gene that codes for an active drug efflux pump P-glycoprotein on the cell surface that decreases cellular accumulation of the drug [5, 6]. ADR resistance may also be attributed to the impaired ability of drug trafficking or altered intracellular distribution within the cell