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Specificity in cancer immunotherapy

Andrea Schietinger^a, Mary Philip^b, and Hans Schreiber^a

^aDepartment of Pathology and Committee on Immunology, The University of Chicago, 5841 S. Maryland Avenue, Chicago, Illinois 60637, USA

^bDepartment of Medicine, The University of Chicago, 5841 S. Maryland Avenue, Chicago, Illinois 60637, USA

Abstract

From the earliest days in the field of tumor immunology three questions have been asked: do cancer cells express tumor-specific antigens, does the immune system recognize these antigens, and if so, what is their biochemical nature? We now know that truly tumor-specific antigens exist, that they are caused by somatic mutations, and that these antigens can induce both humoral and cell-mediated immune responses. Because tumor-specific antigens are exclusively expressed by the cancer cell and are often crucial for tumorigenicity, they are ideal targets for anti-cancer immunotherapy. Nevertheless, the antigens that are targeted today by anti-tumor immunotherapy are not tumor-specific antigens, but antigens that are normal molecules also expressed by normal tissues (so-called “tumor-associated” antigens). If tumor-specific antigens exist and are ideal targets for immunotherapy, why are they not being targeted?

In this review, we summarize current knowledge of cancer-specific antigens: their identification, immunological relevance and clinical use. We discuss novel tumor-specific epitopes and propose new approaches that could improve the success of cancer immunotherapy, especially for the treatment of solid tumors.

Keywords

cancer immunotherapy; tumor antigens; specificity; glycosylation; Cosmc

2. Introduction

The system of positive and negative selection that creates our repertoire of T and B cells evolved to discriminate between “self” and “non-self” structures. There is a fine-tuned balance between immunological “self-tolerance” and “foreign attack” that allows the destruction of “non-self” while preserving “self”. In the case of infectious diseases, microbial organisms express proteins, carbohydrates, and lipids that differ greatly from our own and therefore present a wide array of potential antigens for immune recognition. However, many common cancers in man do not have a viral etiology but are caused by somatic mutations that alter self-proteins through single amino acid substitutions,

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To whom correspondence should be addressed: Hans Schreiber, Department of Pathology and Committee on Immunology, The University of Chicago, 5841 S. Maryland Avenue MC 3008, Chicago, IL 60637, USA, Phone (773) 702-9216, Fax (773) 702-9224, hszz@midway.uchicago.edu.

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truncations, deletions, or fusion to other proteins. If the mutated protein can be effectively presented to T cells or recognized by B cell receptors, cancers can become antigenic and be recognized by the immune system. Epigenetic changes in cancers also lead to over- or aberrant expression of normal proteins.

Based on qualitative differences, non-viral tumor antigens are divided into two classes: (1) tumor-specific antigens that are caused by mutations and (2) tumor-associated shared antigens that result from over- or aberrant expression of non-mutated proteins [1, 2]. Because tumor-specific antigens are exclusively expressed by cancer cells and are often critical for tumorigenicity, they are ideal targets for anti-cancer therapy. However targeting tumor-specific antigens would require therapeutic strategies to be tailored to individual patients or small subgroups of patients, making the targeting of tumor-specific antigens more technically challenging and labor-intensive. As a result, the focus of industry and cancer immunologists has been on targeting peptides from normal molecules that are not tumor-specific but expressed on large groups of cancers. Thus, until now mainly tumor-associated shared antigens have been targeted by active and passive cancer immunotherapy (notable exceptions are discussed by Schmidt *et al.*; Sampson *et al.*; Buckwalter and Srivastava; Lucas and Coulie, in this issue). Here, we highlight the characteristics of tumor-specific antigens, the advantages and disadvantages of targeting these antigens, and new directions that could improve cancer immunotherapies.

3. Identification of tumor antigens

A great deal of evidence in mice and men has demonstrated that the host generates antibodies and T cells against developing tumors. Strategies have been developed to use patients' T cells (CD4⁺ and CD8⁺) and antibodies (immunoglobulin, IgG) for the identification and characterization of tumor antigens.

3.1. Antibody-defined tumor antigens

The investigation of serological responses to tumors has a long history. In the 1970s, Lloyd Old and colleagues established the strategy known as "autologous typing". Serum from a cancer patient was tested to identify IgG-antibodies that reacted against the patient's own cancer cells but not against autologous normal cells, typically fibroblasts [3, 4]. Through autologous typing, Old and colleagues showed the existence of three classes of tumor-antigens. Class 1 antigens are expressed by autologous cancer cells but not by autologous normal control tissues or by allogeneic cancer cells. Therefore, these antigens are truly tumor-specific. Class 2 antigens are found on autologous and allogeneic cancer cells as well as on a subset of normal cells and are therefore tumor-associated shared antigens. Class 3 antigens are cell surface molecules widely expressed by normal or non-malignant cells. While autologous typing strongly suggested the existence of truly tumor-specific antigens, the precise biochemical nature and genetic origin of those antigens remained elusive [5].

Through the application of molecular cloning techniques, SEREX, or serological analysis of autologous tumor antigens by recombinant cDNA expression cloning, has largely replaced autologous typing [6, 7]. This has allowed the direct molecular identification of antigenic tumor proteins. High-titer IgG antibodies from cancer patients are used to probe proteins encoded by tumor-derived cDNA libraries (usually allogeneic cDNA libraries and tumor cell lines) expressed in prokaryotes. Since the establishment of the SEREX database in 1997 and later incorporation of it into the Cancer Immunome Database (<http://www.licr.org/CancerImmunomeDB>), over 2700 clones/sequences have been identified and documented. None of these antigens turned out to be tumor-specific, with the exception of p53 [8], a molecule that was known to be mutated frequently in cancer cells. Generally, SEREX-defined molecules encode normal proteins such as differentiation antigens, embryonic and

fetal proteins, and molecules normally found in spermatogonia (cancer-testis antigens) [9–12]. During tumor development, many self-molecules are up-regulated and elicit humoral or T cell responses, leading some researchers to classify these self-molecules as tumor antigens. However, there is much debate about whether such immune responses are beneficial, insignificant, or even harmful to the cancer patient [13].

SEREX remains the prominent technology for identifying antibody-recognized tumor antigens (<http://www.cancerimmunity.org/SEREX/methodology>), though the method does have drawbacks: (i) Because allogeneic expression libraries are generally used, only targets that are shared between different cancers are detected, thereby excluding most tumor-specific antigens that are not shared. (ii) Since SEREX relies on cDNA expression libraries displayed on phage, antigens that result from alterations in post-translational modifications are not detected. Innovative analytical approaches such as serological proteome analysis (SERPA) [14] and high-throughput protein microarray technology [15] have emerged as methodologies for biomarker discovery. For an in-depth summary of these antigen identification strategies see review [16].

3.2. T cell-defined tumor antigens

Both CD8⁺ cytolytic T cells (CTL) and CD4⁺ T helper cells recognize small peptide antigens in the context of the major histocompatibility complex (MHC) molecule. To identify the genetic basis of CD4⁺ T cell-recognized tumor antigens, one approach has been to identify the sub-cellular compartment containing the CD4⁺ T cell-stimulatory activity, separate the stimulatory protein fraction by reversed-phase high-performance liquid chromatography (RP-HPLC), further separate by SDS gel electrophoresis and T-cell Western, and finally, protein-sequence the stimulatory band. This approach allowed the first molecular and genetic identification of a tumor-specific antigen [17]. Another method of identifying T-cell-recognized antigens is through elution of peptide from MHC molecules of cancer cells. Peptides are eluted from the surface of cancer cells (or from MHC class I or II molecules purified from cancer cells), pulsed onto antigen presenting cells (APC), and tested for reactivity with the patients' lymphocytes. Purification and sequencing of these peptides identifies the parental protein [18–21]. In third approach similar to SEREX, T cell epitope cloning, cDNA libraries generated from tumor cells are transfected into target cells expressing the appropriate MHC Class I or II molecules, and anti-tumor T cells are used to identify the appropriate transfectant [22–26].

4. Tumor-associated antigens

Tumor-associated shared antigens represent a group of normal non-mutant molecules that can be subdivided into four major categories according to expression pattern:

1. *Oncospermatogonial antigens (Cancer testis antigens)* are expressed by cancer cells but are normally found on spermatocytes/spermatogonia (MAGE, GAGE, BAGE, NY-ESO-1). Since testis is considered an immunoprivileged site [27] these antigens were thought not been seen by the immune system. However, some of these antigens are also expressed in the thymus [28]. These antigens, like all other tumor-associated antigens, also induce some levels of central or peripheral tolerance; T and B cells reactive to these self-antigens have lower affinity and avidity unless they are generated as a non-self response in a knock-out mouse.
2. *Differentiation antigens* are molecules expressed on non-malignant cells of the same cell lineage as the tumor (TRP-1, gp100, MART-1, tyrosinase, CD20, EpCAM).

3. *Oncofetal antigens* are antigens found on embryonic and fetal tissues as well as certain cancers (alpha fetoprotein, carcinoembryonic antigen CEA, 5T4).
4. *Over-expressed antigens* are normal proteins whose expression is up-regulated in cancer cells (PSA, wild-type p53, Her2/Neu and EGFR).

Because of their expression in normal, non-malignant tissue, tumor-associated shared antigens are more likely to have induced immunologic tolerance and are less likely to stimulate effective immune responses [29]. Self-reactive T cells are readily deleted and/or functionally inactivated, and when not deleted, have a reduced capacity to recognize target antigens [30]. If a cancer vaccine does break tolerance to a tumor-associated shared antigen and self-reactive T cells are made effective, destruction of normal tissue or even fatal autoimmune damage can result [31, 32]. Though T cells can be engineered to express receptors with high (nanomolar) affinity for self-antigens that are highly expressed in cancers, there is concern that such T cells could cause significant autoimmune damage if used therapeutically. Such autoimmune damage may be tolerable if the self-antigen is expressed only on non-essential normal tissue. For example, passive immunization with monoclonal antibodies (Rituximab, anti-CD20 mAb) directed against CD20, a tumor-associated shared antigen frequently expressed on non-Hodgkin's lymphoma has been successful [33], because the deletion of normal B cells that occurs as a consequence of effective anti-CD20 treatment can be tolerated. When the lymphoma relapses normal B cells usually reappear as well. It is unclear, however, whether T cells that target self-antigens on tumors could be similarly effective, even if normal cells expressing those antigens were dispensable.

5. Tumor-specific antigens

Cancer results from the accumulation of somatic mutations, and cancer cells contain a large number of mutant proteins [34, 35]. Which mutant proteins will be recognized as tumor-specific antigens in an individual patient depends upon the particular repertoire of MHC Class I and II molecules and T or B cell receptors in that patient. With the extremely large number of mutations found in common human cancers [36–38], one would expect that every human cancer cell harbors at least a few mutations that can be therapeutically exploited.

The first evidence for tumor-specific immune responses was provided by early transplantation experiments using chemically or UV-induced murine tumors in autochthonous hosts (the same host in which the tumor had originated) or syngeneic (genetically-identical) hosts [39, 40]. These studies revealed that transplanted tumors elicited strong immune responses that were specific for the individual tumor [40–42]: immunization with a given tumor gave rigorous protection only against re-challenge with the same tumor but not other tumors. These experiments led to the conclusion that each cancer was “unique” and displayed individually distinct antigenic determinants.

Over the last 10 years, the genetic origins of several tumor-specific antigens from spontaneous or induced cancers in mouse and man have been identified, and in each case, the antigen was caused by a somatic mutation resulting in a change of protein sequence [17, 26, 43–47]. Only a relatively small number of cancers have undergone this careful analysis, and most of the mutations identified were found in one individual tumor but not in cancer cells from other patients [48–52]. However, once more cancers are analyzed, prominent target genes and mutation patterns will likely emerge. For example three *Ki-RAS* point mutations (single amino acid substitutions) are found in about 95% of all patients with pancreatic cancers. The majority of the tumor-specific antigens so far identified result from mutations in genes encoding intracellular proteins.

Tumor-specific antigens are ideal targets for cancer immunotherapy because they are exclusively expressed by the cancer cell and not on non-malignant tissues, minimizing the risk of autoimmune destruction. In addition, because the immune system has not been previously exposed to these antigens, there is no neonatal or peripheral tolerance to these antigens prior to tumor development. During tumor development, the immune system can recognize these determinants as non-self and generate specific high-affinity antibodies and T cells against them. In contrast, T and B cells with reactivity to self-antigens generally have low affinity and are susceptible to immunological tolerance particularly if the peptide has a good affinity to the presenting MHC molecule [53].

Another major advantage of tumor-specific antigens is that many of the mutant proteins that result in tumor-specific antigens are themselves essential for tumorigenesis. Several studies have reinforced the importance of targeting such essential proteins with cancer immunotherapy [52, 54–57]. Experimental and clinical observations suggest that those proteins required for the maintenance of the malignant phenotype are less likely to get lost during tumor progression, even under the selective pressure of anti-cancer treatments such as immunotherapy [58, 59]. Some of these proteins are fusion proteins such as those encoded by the chimeric *BCR/ABL* gene in CML or the chimeric *EWS/ATF1* genes in clear cell sarcomas. Others are point-mutated tumor-suppressor genes such as L26. In contrast, targeting of tumor-associated shared antigens that are non-essential can lead to the emergence of antigen loss variants [60–63].

Cancer cells express both tumor-associated shared antigens and tumor-specific antigens [64]. Our group and others have shown in various experimental models that there is a hierarchy in the host immune response to different epitopes such that certain antigens are “immunodominant” while others are “immunorecessive” [65, 66]. Tumor-specific epitopes were found to be immunodominant, while tumor-associated shared antigens were immunorecessive. Immunorecessive antigens could only induce an immune response once the immunodominant epitope was lost. Immunodominance of tumor-specific epitopes was also demonstrated in humans; Lennerz et al. revealed recently that the immune response in a melanoma patient was dominated by CD8⁺ T cells against mutated neo-antigens over CD8⁺ T cells recognizing shared antigens [56].

All the qualities of tumor-specific antigens discussed above (i) immunogenicity, (ii) decreased risk of inducing autoimmunity, (iii) decreased risk of immune escape, and (iv) immunodominance, make tumor-specific antigens attractive targets for immunotherapy. However, unlike tumor-associated shared antigens, tumor-specific antigens are expressed only on individual patients' cancer cells or small subsets of tumors and thus require the development of personalized therapy for individual patients. For the most part is too costly and time-consuming to be feasible currently. However, rapid advances in technology over the last decade have decreased the cost and time to identify not only mutations and antigens but also identify the specific MHC molecules expressed by each patient. Continued improvements and the exponential increase in available “omics” technology and database information will soon make such individually-tailored therapies a reality.

5.1. Glycosylation – a post-translational modification

Until recently, the tumor-specific tumor antigens that have been identified in mouse and human are mutant peptide epitopes. In eukaryotic cells, 95% of all proteins are post-translationally modified. Post-translational modifications such as phosphorylation, glycosylation, nitrosylation, ADP-ribosylation, ubiquitination, and acetylation occur after protein synthesis in the ER and Golgi. These alterations affect the solubility, folding, localization, half-life and biological functions of proteins.

Glycosylation is the most frequent post-translational modification found in eukaryotes, and it is estimated that 50–80% of cellular proteins are glycosylated. In addition to membrane proteins, cytosolic and nuclear proteins are also glycosylated. Since glycoproteins, carbohydrates and glycolipids are the most abundant structures present on the surface of eukaryotic and prokaryotic cells, they are the first structures encountered by the immune system and critical in primary immune recognition. The hallmarks of the adaptive immune response are the ability to recognize a wide range of antigens ($\sim 10^9$ epitopes), to respond to new antigens that have not yet been seen in the evolutionary history of the species, to remember which antigens have been encountered (immunologic memory), and to discriminate between self and non-self. Initially, it was thought that only pure protein epitopes could be presented on MHC and induce T cell responses [67]. This was due in part to the observation that immunization with carbohydrate antigens usually resulted in low-affinity IgM responses without memory. To obtain a strong immune response, as reflected by IgG production, both T and B cells are required. However, recent publications have demonstrated that non-peptide molecules such as pure carbohydrates, glycopeptides and glycolipids can be presented on MHC molecules and recognized by T cells [68–78]. Although pure peptide epitopes are still considered the primary targets for T cell responses, there is growing recognition that glycopeptides also induce T cell responses [71–73, 75–77]. Therefore, the peptide epitopes that have been identified thus far as tumor epitopes thus far might represent only a small fraction of potential targets.

There are two types of glycosylation, N- and O-glycosylation. N-glycosylation occurs at the amino acid asparagine (Asn). The consensus sequence for N-glycosylation is the presence of the amino acid sequence Asn-X-Serine/Threonine (X may be any amino acid except Proline). O-glycosylation occurs at Serine (Ser) or Threonine (Thr) residues, but despite much effort, no consensus sequence for O-glycosylation has been identified. The enzymes responsible for glycosylation, glycosyltransferases and glycosidases, recognize specific sugars, sugar sequences, and often peptide moieties of substrates, and with very few exceptions, each type of enzyme synthesizes only one linkage. On the other hand, a particular linkage may be synthesized by one or more members of a glycosyltransferase family. While the initial steps of glycosylation are carried out in the endoplasmic reticulum (ER), subsequent steps occur in the Golgi. Glycosyltransferases are arranged in assembly line-fashion in the Golgi, with early-acting enzymes localized in the cis-Golgi, intermediate-acting enzymes in the medial-Golgi, and those adding terminal structures in the trans-Golgi. N-glycosylation is a complex biochemical process carried out in three major steps [79] (Figure 1). First, a common complex carbohydrate “stump” of 15 monosaccharides (Glc3Man9GlcNAc2) is transferred from a dolichol-linked donor to Asn residues as proteins enter the lumen of the ER. In the cis-Golgi, this carbohydrate stump is trimmed and processed by specific glycosidases. This is followed by the addition of further monosaccharides by N-acetylglucosamine-transferases in the medial-Golgi. The attachment of terminating sugars (sialic acid, fucose, sulfate or galactose) in the trans-Golgi results in high molecular-weight, highly-branched sugar chains (Figure 1).

In contrast, O-glycans are assembled by stepwise addition of single monosaccharides; each sugar is transferred from a nucleotide sugar donor by specific membrane-bound glycosyltransferases. The initial step is carried out in the cis-Golgi and usually involves the linkage of N-acetylgalactosamine (GalNAc) (especially in mucin type glycoproteins) or N-acetylglucosamine (GlcNAc) (in nuclear glycoproteins) to the hydroxyl group of Ser or Thr residues (Figure 1) [80]. However, alternative pathways have been described (e.g. O-glucose, O-mannose or Lewis-structures). Next, galactose (Gal) is added to GalNAc to form the Core 1 carbohydrate structure, the simplest disaccharide structure found in O-glycans. Core 1 is the base on which branched Core 2 O-glycans or other higher molecular-weight structures are formed. Ultimately, the sugar chain is terminated with the addition of distinct

monosaccharides and molecules that block further elongation: sialic acid, fucose, galactose or sulfate groups. There are at least 8 O-Core structures (Figure 1); Cores 1–4 are most common in mammals. O-linked carbohydrates tend to be shorter and simpler structures than N-linked carbohydrates, but because of their greater heterogeneity, O-glycans have not been as well studied as N-glycans.

The field of glycomics has lagged behind genomics and proteomics because of the incredible variety of carbohydrate structures that result not only from the different sugar components, but also the multiple different glycosidic linkages that are possible [80, 81]. This is in contrast to the linear, single-linkage construction of nucleic acids and proteins. Also, unlike nucleic acid and proteins, the biosynthesis of carbohydrates is not “template-driven,” making their analysis even more challenging. These factors have made high-throughput analysis of carbohydrate structures difficult. More recently, the combination of several biochemical analytic methods, such as mass spectrometry (MS), chromatography, enzymatic, and chemical approaches has made it possible to unveil the changes in carbohydrate structures and their relation to biological function and disease. Quantitative and qualitative changes in glycosylation have been associated with several diseases including cancer [82–84], autoimmune diseases such as rheumatoid arthritis, lupus, [85, 86], cystic fibrosis [87–89] and others [90, 91].

5.2. Altered glycosylation in cancer

Altered glycosylation is one of the hallmarks of cancer cells (for review see [80, 83, 92, 93]). Changes in cell surface carbohydrate structures, such as over- or under-expression of sugar moieties and neo-expression of certain carbohydrate structures occur during tumor progression, invasion and metastasis [94, 95]. Such aberrations occur in both N- and O-linked glycosylation. Altered N-glycosylation leads to an increase in complex, highly 1,6-branched N-glycans in several cancers [81, 94, 96]. This is due to overproduction of the enzyme glucosaminyl transferase-V (GnT-V) and correlates with poor prognosis and decreased survival [97, 98]. In contrast to N-glycosylation, O-glycosylation in cancers is often reduced, leading to the accumulation of simpler, Core 1-based O-glycans. O-glycan epitopes commonly found on cancers include the disaccharide Gal β (1 $\rightarrow$ 3)GalNAc [T, TF (Thomsen-Friedenreich)] [99, 100], the monosaccharide GalNAc [Tn (“T nouvelle”)] [101], the Lewis^x or ^a antigens [102], and their sialylated counterparts sialyl-T, sialyl-Tn and sialyl-Lewis^x or ^a antigens [103] (Figure 2). Because these O-glycans are shared by various malignancies, they are referred to as tumor-associated carbohydrate antigens (TACA) [80, 92, 104] (Figure 2). In most cases, these quantitative and qualitative changes result from over- or under-expression of specific transferases and glycosidases secondary to epigenetic changes. For example, reduced activity of certain enzymes has been associated with decreased transcription caused by methylation in CpG islands of promoter DNA regions [105]. There was much interest when certain tumor-associated sugar epitopes were shown to be actively involved in metastasis by facilitating intravasation of cancer cells, binding of the circulating cancer cells to the endothelium of distant tissues, extravasation, and colonization at distant sites [106–108].

Due to their broad expression profile in several malignancies, much effort has gone into targeting TACAs with active and passive immunotherapy [83, 92] and trying to augment their antigenicity and immunogenicity [109–113]. In addition to TACAs, gangliosides (GD3, GD2 and GM2) have also emerged as promising monoclonal antibody (mAb) targets for various cancers such as melanoma and neuroblastoma [92, 110, 114].

Since pure carbohydrate epitopes are unlikely to stimulate T helper cells, they generally elicit only low-affinity IgM, and not IgG [109]. It is therefore necessary to resort to allo- or xenogeneic immunization and/or conjugation to carrier molecules to generate anti-

carbohydrate mAb with higher affinity. However, these pure carbohydrate epitopes are also found in embryonic and normal adult tissues [92], and are thus tolerogenic. However, abnormal truncation of carbohydrate residues can unmask peptide epitopes, which then become antigenic, e.g. MUC1 [115–117]. The expression of distinct carbohydrate structures associated with particular proteins has also been reported. However, these novel epitopes have also been found to be expressed in embryonic tissues (oncofetal antigens): prostate-specific antigen PSA [118, 119], carcinoembryonic antigen CEA [120–122] and fibronectin [123]. Consequently, T cells or B cells expressing high-affinity T cell receptors and antibodies respectively for these antigens are likely eliminated during embryonic development and therefore not present during tumor development [124].

5.3. A mutation in the chaperone *Cosmc* converts a wild-type protein into a tumor-specific antigen — prototype for a new class of truly tumor-specific antigens

Recently, we reported that a wild-type protein can be converted into a tumor-specific cancer target molecule [38]. Our work began with the observation that a syngeneic, high-affinity monoclonal antibody, 237mAb, showed absolute specificity for a spontaneous murine fibrosarcoma, Ag104A. The antibody did not bind to a second spontaneous tumor or autologous normal tissue from the same mouse, or any other tumor cell line tested [125]. Through immunoaffinity purification and protein sequencing, the antigen was identified as the highly O-glycosylated, type I transmembrane protein OTS8 (identical to Podoplanin, Aggrus, PA2.26). Interestingly, 237mAb only recognized OTS8 expressed in Ag104A, but not OTS8 expressed in other cell lines. Since the tumor-specific antigens so far identified were found to be mutant proteins, we were surprised when sequencing of Ag104A-OTS8 cDNA did not reveal any mutations. This led us to hypothesize that the unique specificity of the antigen was the result of an Ag104A-specific post-translational modification. We demonstrated that altered glycosylation was responsible for the 237mAb epitope and that a particular amino acid, Thr 77 in the OTS8 extracellular domain, carried the antigenic sugar epitope. Biochemical analysis revealed that OTS8 from Ag104A did not display the normally-associated Core 1 structure (Gal β (1 $\rightarrow$ 3)GalNAc), but instead had the truncated O-glycan Tn (GalNAc). In humans, the enzyme catalyzing the transfer of galactose from UDP-galactose to GalNAc-S/T is a type II transmembrane protein, Core 1 β 3galactosyltransferase (C1 β 3GALT). Interestingly, enzymatic activity was shown to require the co-expression of a chaperone protein, *Cosmc* (Core 1 β 3Gal-T-specific molecular chaperone) [126]. *Cosmc* was found to be mutated in the human T leukemia Jurkat cell line, causing loss of C1 β 3GALT activity, loss of Core 1 structures, and overexpression of Tn [126, 127]. Understanding the regulation of this enzyme is of great interest because truncated Tn and sialyl-Tn motifs are known to be tumor-associated antigens in human breast and colon cancers [92, 99, 101, 128–131]. In addition, Tn-related autoimmune diseases such as IgA nephropathy [132], Tn syndrome [133, 134], and Henoch-Schoenlein purpura [135] are thought to result from diminished or abnormal C1 β 3GALT reactivity. Tn syndrome is caused by a somatic mutation in *Cosmc* [91]. We showed that in Ag104A, a somatic mutation in *Cosmc* abolished C1 β 3GALT, disrupting O-glycan Core 1 synthesis, resulting in over-expression of the truncated form GalNAc (Tn), and ultimately creating a tumor-specific glycopeptidic neo-epitope on wild-type OTS8 (Figure 3). The novel combination of the monosaccharide GalNAc with a 10 amino acid peptide sequence of the extracellular domain of OTS8 created a truly tumor-specific antigen, which induced the generation of the syngeneic, high-affinity, highly-specific, and therapeutically potent monoclonal antibody, 237mAb. Antibody specificity for glycopeptidic epitopes can arise in two ways: the antibody can directly bind both to amino acids residues and sugar groups, or carbohydrate moieties can alter protein folding in a way that allows antibody binding. In the case of the 237mAb epitope, it is not yet known which mechanism is at work.

While mutations that change the amino acid sequence of a single protein create only one potential antigen, mutations such as the *Cosmc* mutation alter glycosylation globally on cell surface proteins [38], creating many potential tumor-specific glycopeptidic neo-epitopes which can be targeted by mAbs. Given that GalNAc (Tn) is the precursor for longer, more complex O-glycan structures, it might be expected that such neo-epitopes would be detected as biosynthetic intermediates in normal cells, and thus induce tolerance. However, this does not in fact occur because the addition of subsequent carbohydrate moieties to GalNAc happens extremely rapidly in the cis-Golgi [127, 136]. These “intermediate” glycopeptides are not seen by the immune system during embryogenesis or adulthood, but when created during tumor development, they become highly antigenic. Glycopeptidic neo-epitopes not only represent potential targets for antibody therapy, but may also be targeted by T cells, since glycopeptides can be presented on MHC. Mutations in *Cosmc* have now been found in other murine (Ag104A, Neuro2A) and human tumors (LSC, Jurkat) as well as in patients with the autoimmune disease Tn syndrome, suggesting that this pathway may be commonly targeted in cancer cells.

The 237mAb story exemplifies the importance of aberrant post-translational modification in the creation of tumor-specific antigens. The current framework for antigen identification: mutant gene → mutant protein → tumor antigen may be overly simplistic. The paradigm must be expanded because a mutation in one protein can have a domino effect, affecting wild-type proteins downstream of the actual mutation (Figure 4). Potentially any mutation in an upstream protein, such as glycosylation enzymes or proteins responsible for other post-translational modifications can create “neo-epitopes” on wild-type proteins. Because these changes in post-translational modification are due to tumor-specific mutations, they are likely to be tumor-specific and neither tolerogenic, nor cause autoimmunity when targeted. The difficulty in identifying these types of tumor-specific targets arises in part from the intrinsic limitations of methodologies currently in use. For example, SEREX relies on cDNA expression libraries displayed on phage and cannot detect antigens created by post-translational modifications. However, even if eukaryotic systems are used to search for post-translationally modified tumor epitopes, one must consider that many proteins may only become antigenic when expressed by the actual cancer cell of interest if due to cancer-specific alterations in post-translational modification. The existence of a tumor-specific antigen such as the 237mAb epitope should not only prompt the search for other such tumor antigens but also the development of new methodologies to identify such tumor-specific antigens. This will greatly expand the pool of potential tumor antigens which can be used as targets for the diagnosis and treatment of cancers.

6. Conclusion - Cancer therapy, getting personal

It has been shown that tumor-bearing hosts develop specific CD4⁺ and CD8⁺ T cells and antibodies to tumor-specific antigens [1, 10, 17]. However, most tumor-specific antigens have been found to be individually-restricted and not expressed on other malignancies and patients. While there has been some success in targeting shared antigens, the reasoning and experimental evidence to support the targeting of tumor-specific antigens is strong and should prompt the development of individualized/personalized therapy targeting tumor-specific antigens.

Over the last decade, anti-cancer therapies have become more and more selective in an attempt to overcome tumor resistance and minimize toxic side effects. New anti-cancer drugs such as the tyrosine kinase inhibitor imatinib [137] is just the first of many molecular, targeted therapies in use or in the pipeline.

The success seen thus far in experimental and clinical settings targeting tumor-specific antigens should spur the further application of high-throughput technologies to the individual analysis of a patient's serum and T cell specificities in order to develop tailored immunotherapy. Cancer immunotherapy is an ideal area for the development of personalized medicine that will allow us to take advantage of tumor-specific antigens for the diagnosis and treatment of cancers.

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Figure 1. Biosynthesis of N- and O-glycans. N-glycosylation occurs in three major steps in the ER and the Golgi resulting in long, branched sugar chains. O-glycosylation occurs by the stepwise addition of monosaccharides and begins with the linkage of N-acetylgalactosamine (GalNAc) to Ser or Thr. Further elongation leads to the formation of so-called Core structures (Core 1–8). Adapted from A. Varki *et al.*, Essentials of Glycobiology, 1999.

Figure 2. Tumor-associated carbohydrate antigens (TACA). Shown are the most common carbohydrate structures known to be overexpressed by malignancies: Tn, T (TF, Core1), Sialyl-Tn, Lewis^a, Sialyl-Lewis^a, Lewis^x, Sialyl-Lewis^x (right panel). Left panel shows legend of carbohydrate symbols.

Figure 3.

A somatic mutation in the chaperone Cosmc leads to the creation of a tumor-specific, glycopeptidic neo-epitope on a wild-type protein. Mutation of the chaperone gene, Cosmc, abolished the activity of the Core 1 $\beta(1,3)$ galactosyltransferase needed for the linkage between N-acetylgalactosamine (GalNAc) and Galactose (Gal) (= Core 1). This disrupted O-glycan Core 1 synthesis, leaving only GalNAc attached to the Thr residue. The novel combination of the monosaccharide GalNAc with a 10 amino acid wild-type protein sequence RGTKPPLEEL from the transmembrane protein OTS8 creates a tumor-specific antigen.

How to create a Tumor-specific Antigen

Figure 4.

Pathways of tumor-specific antigen creation. On the left is shown the previous paradigm for tumor-specific antigen creation. On the right is the new pathway; in which a mutation in an upstream gene causes aberrant post-translational modification and converts a wild-type protein into a tumor-specific antigen.