Review

New Concepts in Tumor Antigens: Their Significance in Future Immunotherapies for Tumors

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The identification and molecular characterization of self-antigens expressed by human malignancies that are capable of elicitation of anti-tumor immune responses in patients have been an active field in tumor immunology. More than 2,000 tumor antigens have been identified, and most of these antigens are self-antigens. These significant progresses have led to the renaissance of tumor immunology and studies on anti-tumor immunotherapy. However, despite of the progress in the identification of self-tumor antigens, current antigen-specific immunotherapies for tumors are far less satisfied than expected, which reflects the urgent need to improve our understanding on self-tumor antigens. In order to develop more effective antigen specific anti-tumor immunotherapies and to monitor the responses to these immunotherapies in patients with tumors, many important fundamental questions need to be addressed. We propose for the first time that the studies in addressing the characteristics of self-tumor antigens and autoantigens are grouped as a new subject termed "antigenology". In this brief review, we would outline the progress in the identification of tumor antigens in solid tumors and hematologic malignancies, and overview the new concepts and principles of antigenology and their significance for future immunotherapies to these malignancies. *Cellular & Molecular Immunology*. 2005;2(5):331-341.

Key Words: tumor, tumor antigen, new concept, anti-tumor immune response, immunotherapy

Introduction

The identification and molecular characterization of self-antigens expressed by human malignancies that are capable of elicitation of anti-tumor immune responses in patients have been an active field in tumor immunology (1). As often occurred in other fields, such as regulatory T cells (2), there was no exception in tumor immunology that skepticism was high regarding whether or not immune response to tumors could exist in humans, and whether anti-tumor immune responses could ever be used to develop effective immunotherapy against tumors. In the earlier years, a representative opinion on tumor immunotherapy concluded that: "It would be as difficult to reject the right ear and leave the left ear intact as it is to immunize against cancers" (3, 4). The assumption underlying this comment turned out to be wrong since it assumed that similar to the left ear and right ear, there were no antigenic differences between normal tissues and cancers. In 1985, Rosenberg's team reported that the administration of high dose recombinant interleukin-2 (IL-2) to humans mediated the regression of bulky, invasive tumors in selected patients with metastatic melanoma, kidney cancer and non-Hodgkin's lymphoma, which clearly demonstrated that anti-tumor immune responses enhanced by IL-2 could eradiate tumors (5). Much has changed in the last twenty years due to the significant progress in immunology, molecular biology and completion of human genome sequencing (1, 4, 6, 7). The substantial antigenic differences have been found between tumors and normal tissues. A milestone in tumor immunology was the cloning of tumor antigen MAGE-1 by Boon's team in 1991 (6, 8, 9), and subsequent characterization of the first HLA-restricted T cell defined antigenic epitope a year later (10). In 1995, another breakthrough was reported, Pfreundschuh's team developed a new method of serological cloning approach called SEREX (serological analysis of tumor antigens by screening the recombinant cDNA expression libraries with sera from cancer patients) (1, 11-13). It allows a systemic and unbiased search for antibody responses against protein antigens expressed by human tumors. More than 2,000 tumor antigens have been identified (1) (also see an excellent database http://www.cancerimmunity.org/statics/databases.htm). These

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significant progresses have led to the renaissance of tumor immunology and studies on anti-tumor immunotherapy (8, 11). However, despite of the progress in the identification of self-tumor antigens, current antigen-specific immunotherapies for tumors are far less satisfied than expected, which reflects the urgent need to improve our understanding on self-tumor antigens. In order to develop effective antigen specific anti-tumor immunotherapies and to monitor the responses to these immunotherapies in patients with tumors, many questions need to be addressed including: 1) What are the mechanisms underlying the selection of these self-proteins but not the other proteins to become tumor antigens? 2) What are the signals controlling the expression of these tumor antigens? 3) What are the immunodominant epitopes in these tumor antigens? 4) What are the mechanisms underlying the differences in immunogenicity of tumor antigens among patients? 5) What are the correlations between the kinetics of tumor antigen expression and tumor growth and the responses of tumor to immunotherapy? We propose for the first time that the studies in addressing these important issues are grouped as a new subject termed "antigenology". In this brief review, we would outline the progress in the identification of tumor antigens in solid tumors and hematologic malignancies, and overview the new concepts and principles of antigenology and their significance for future immunotherapies to these malignancies. We apologize for failing to cover many invaluable reports, reviews and opinions in the field due to the space limit of this review.

Do tumor antigen-based immunotherapies really hold a promise for the development of novel immunotherapies to malignancies?

In addition to cell therapy with patients' cells transfected with cytokines, or T cell co-stimulation molecules B7 (14), adoptive therapy, dendritic cell therapy, heat shock protein preparation therapy, or exosome preparation therapy, antigen-specific immunotherapy has always been desirable since it promises to offer higher efficacy and lower side-effect in eradication of tumors. Based on the effective mechanisms, tumor antigen-specific immunotherapy can be classified into antibody anti-tumor immunotherapy (15) and T cell anti-tumor immunotherapy (16). Due to its intrinsic limitation of antibodies in penetration of cells, antibody anti-tumor immunotherapy has been targeted to tumor antigens expressed on cell surface (15), for example, antibody therapies of acute myeloid leukemias have targeted to the cell surface antigens CD13, CD15, CD33, CD45, and CD66 (17). In 1997, the US Food and Drug Administration approved Rituximab (a B cell surface antigen) for the treatment of relapsed/refractory low grade or follicular $CD20^+$ B cell non-Hodgkin's lymphoma. After conducting 41 studies in using Rituximab to treat a total of 2,170 patients with follicular lymphoma, the results showed that monotherapy with Rituximab led a 50% response rate but less than a 10% clinical remission (CR) (18). When Rituximab was combined with chemotherapy, its efficacy was enhanced with a responses rate of 81-97% and a 35-74% CR (19). In addition, Rituximab has also been used in the treatment of multiple myeloma, and Waldenstrom's macroglobulinemia (20), Castleman's lymphoproliferative disorder (21), gastric diffuse B cell lymphoma (22), and mantle cell lymphoma (23). By several different mechanisms, Rituximab can selectively deplete B cells, which include: (a) antibody dependent cell mediated cytotoxicity, ADCC; (b) complement-dependent cytotoxicity, CDC; and (c) inhibition of cell proliferation and direct induction of B cell apoptosis (19, 24-27). Similar to Rituximab, a phase I study was conducted in patients with advanced solid tumors using the ScFv(FRP5)-ETA, a recombinant, single-chain (sc) antibody toxin with binding specificity for a cell surface tumor antigen HER2 linked to *Pseudomonas* exotoxin A (ETA) (15). Remarkable objective responses were observed, and a fraction of patients experienced a long-lasting complete responses (28, 29).

In addition, studies on identification of HLA-restricted T cell antigen epitopes of tumor antigens and T cell based immunotherapy to tumors have also made significant progress (30). By 2004, more than 257 HLA class I-, and HLA class II-restricted T cell antigen epitopes have been identified (http://www.istitutotumori.mi.it/INT/AreaProfessionale/Human_Tumor/default.asp?LinkAttivo=17B). Since they are derived from various tumors, these T cell antigen epitopes are very useful in diagnosis, prognosis, and immunotherapy to tumors. Furthermore, clinical studies of several formats of active immunization (recombinant viruses, naked DNA, dendritic cells pulsed with peptide, and peptides) in patients with melanoma showed that after two courses of immunization with the gp100, MART-1, or tyrosinase tumor antigens, up to 1-2% of all circulating CD8 T cells had anti-tumor activity, which is several hundred or thousand folds higher than the frequencies of any given antigen-specific T cells in normal T cell repertoire. In combination with IL-2 therapy, the response rate could reach as high as 32% (30). Finally, a team at Memorial Sloan-Kettering Cancer Center in New York reported on 14 patients with chronic myelogenous leukemia (CML) in a phase II study with Bcr-Abl peptides (a CML-specific fusion gene encoded by Philadelphia chromosome) that were given 5 injections or 6 peptides over 10 weeks. A decrease in the percentage of Philadelphia chromosome + (Ph^+) cells (a CML-specific fusion chromosome) was noted in 4 patients (31-33).

Taken together, despite some documented disappointing results (34), these examples have clearly demonstrated that identification of tumor antigens leads to the development of future antigen-specific immunotherapy to tumors. Proof of principle pre-clinical studies have been or will soon be translated into the clinic (16).

What are the major techniques in the identification of tumor antigens and their potential in clinical applications?

Although many antibody binding epitopes (about 15 amino acids) in self-antigens have been characterized, however, one does not have to map the antibody binding epitopes before evaluation of antigen-specific antibody responses. In contrast, before examination of antigen-specific T cell responses, one has to know (a) whether the antigen of interest encodes HLA-restricted T cell antigen epitopes (9-11 amino acids for HLA class I-restricted epitopes; 15-20 amino acids for HLA class II-restricted epitopes); (b) where the epitopes are located in the antigen sequence; and (c) what HLA allele restrictions of the antigen epitopes are. Therefore, it becomes necessary to identify HLA-restricted T cell antigen epitopes of any antigen of interest. In the following section, we have outlined the principles of several major techniques:

T cell epitope cloning: Many antigens recognized by $CD8^+$ T cells have been identified by transfecting cDNA libraries from tumor cells into target cells expressing the appropriate HLA molecule, and then using anti-tumor T cells isolated from tumor infiltrates (TILs) to identify the antigen epitopes presented by HLA on the surface of cDNA libraries-transfected target cells (6, 9, 35) (also see a database of T cell antigen epitopes at http://www.istitutotumori.mi.it/INT/AreaProfessionale/Human_Tumor/default.asp?LinkAttivo=17B). In contrast to solid tumors (9), such as melanoma, for hematologic malignancies, it is hard to isolate TILs to establish tumor-specific T cell lines. In addition, this method is a labor-intensive process and requires T cell culture and cloning expertise.

HLA-binding peptide elution: Peptides eluted from cancer cells or from HLA molecules purified from cancer cells can be pulsed onto antigen presenting cells (APCs) and tested for reactivity with specific anti-tumor lymphocytes. Purification and sequencing of these peptides can then lead to the identification of the parent protein antigens (36, 37). This method requires the protein chemistry expertise in peptide purification and high power mass spectrometry.

SEREX: To identify tumor antigens recognized by the antibody repertoire of cancer patients, in 1995, Pfreundschuh's team developed a new method of serological cloning approach called SEREX (1, 11-13), which allows a systemic and unbiased search for antibody responses against protein antigens expressed by human tumors (also see an excellent online review at http://www.cancerimmunity.org/SEREX/index.htm). The respective tumor antigens in the recombinant cDNA libraries, constructed from fresh tumor specimens and cloned into λ phage expression vectors, are identified from their reactivity with antibodies in the autologous and allogeneic sera of cancer patients. The tumor antigens eliciting high titers of IgG antibody responses (1:500 to 1:1,000) are likely to be a group of T cell-dependent antigens capable of triggering integrated T cell immune responses and antibody responses rather than T cell-independent antigens, which only elicit low titers of T cell-independent natural IgM autoantibodies (38). Elicitation of high tittered IgG antibodies, rather than IgM antibodies, requires the Ig switching process facilitated by T cell help (7, 39). The advantages of SEREX include rapid identification of multiple tumor antigens, no need for establishment of tumor cell lines and pre-established CTL clones (1). SEREX defined tumor antigens are collected in a SEREX database in the Ludwig Institute for Cancer Research website (http://www2.licr.org/CancerImmunomeDB/). Before any claim of identification of novel SEREX is made, the blast search of the NCBI-GenBank/NIH database and the SEREX database with antigen sequence(s) should be performed. The relevance of these SEREX defined tumor antigens to anti-tumor immune responses has been clearly demonstrated in recent reports:

a) SEREX antigens MAGE and NY-ESO-1 are recognized by both T cells and antibodies in the same cancer patient (40, 41);

b) There is good correlation between the antibody titers of cancer-testis (CT) antigen NY-ESO-1 in serum and tumor burden: a positive correlation also exists between NY-ESO-1 antibody titer in serum and specific $CD8^+$ T-cell reactivity (42). These results suggest that integrated humoral and cellular immune responses to cancer-testis like antigens are truly tumor- associated (43);

c) High tittered IgG immune responses to SEREX antigens CML66L and CML28 are associated with the remission of chronic myelogenous leukemia (CML) in patients who responded to donor lymphocyte infusion (DLI) therapy after allogeneic bone marrow transplantation (allo-BMT) (44-48). The SEREX technology can be applied in many laboratories since it requires routine molecular cloning techniques and can be easily modified into evaluation method of patient-oriented anti-tumor immune responses, in particular, once protein microarrays become available.

In addition, since the overexpression of proteins in tumor cells may be the mechanism for the immunogenicity of non-mutated protein antigens, people have also searched proteins or genes encoding the proteins overexpressed in tumors, detected by the techniques including differential display, serial analysis of gene expression (SAGE), or microarray, for tumor antigen candidates (49). The T cell antigen epitopes from these candidate antigens can be further characterized through reverse immunology approach (50).

Why should we go through "all the trouble" to do reverse immunology?

The method of epitope deduction, sometimes called "reverse immunology", postulates that candidate peptide epitopes presented by HLA for stimulating T cells can be identified based on predicted binding affinities of peptide to MHC and scrutinized for immunogenicity based on the functional capacity of experimentally identified epitope-specific T cells (also see several excellent reviews for the details) (6, 9, 35, 50). Traditional approach in T cell antigen epitopes requires the dissection of anti-tumor T cell immune responses in patients, which become a major limitation to the application of cellular tumor immunology strategies beyond melanoma to the majority of common tumors including hematologic malignancies. The reason is that in the majority of tumors other than melanoma, anti-tumor immunoreactivity in

patients is weak (50). Therefore, the reverse immunology becomes a useful alternative for the traditional approach in characterization of antigen-specific T cell responses for the large numbers of SEREX antigens.

Of note, numerous bioinformatic algorithms have significantly contributed to this approach, including the algorithms for the prediction of HLA binding of epitopes, the algorithms for the prediction of proteasome processing of epitopes, and the algorithms for transport associate protein (TAP) binding (also see those useful algorithms in the website resources at http://www. cancerimmunity.org/statics/databases.htm). The identification of HLA class I-, and HLA class II-restricted T cell antigen epitopes from tumor antigens allows us to develop new peptide or DNA vaccines and examine antigen-specific T cells for diagnosis and prognosis. $CD8^+$ cytotoxic T lymphocytes can lyse tumor cells directly, and destroy large tumor masses *in vivo* (51). Immunization using dominant antigenic peptides has been most effective in patients with tumors (52) and has generated surprisingly high levels of circulating T cells directed against tumor antigens with a therapeutic outcome (4). Thus, immunodominant epitopes capable of eliciting remarkable $CD8^+$ T cell responses would contribute decisively to the improvement of peptide-based immunization protocols for patients with tumors (53). Moreover, the majority of MHC class I-restricted tumor antigens identified to date are non-mutated self-proteins or peptides (4, 51), including all the cancer-testis antigens (54) and nonconventional antigens (55). Furthermore, $CD4^+$ T cells have been shown to play an important role in CML remission induced by donor $CD4^+$ T cell infusion for the relapsed CML after allogeneic bone marrow transplantation (56). Also, HLA class II-restricted $CD4^+$ T cells have been demonstrated to recognize mutated tumor antigens (51). Similar to the "traditional" immuno-techniques that are used in detect the antigen-specific antibody responses in patients with tumors (44, 45), such as ELISA (57), phage plaque assay, immunofluorescence, and Western blot, the new developed, epitope based antigen-specific T cell assays including MHC tetramer assay (58-60), and ELISPOT (61) are also available for use in the clinic for evaluation of tumor antigen-specific T cell responses.

How many types of tumor antigens have been identified?

People often incorrectly assume that tumor antigens have to be derived from viruses. Actually, the overlap between infection and cancer is clear with 10-20% of cancers, including Epstein-Barr virus (EBV)-associated lymphoma and a subset of Hodgkin's disease (62), *Helicobacter pylori* infection associated gastric cancer and lymphoma (31, 63), Schistosomes associated with bladder cancer, and liver flukes associated with cholangiocarcinoma (30). Indeed, a small subset of tumor antigens identified are derived from infected viruses, including human papilloma (HPV) E6/E7 associated with cervical cancer, penile cancer and anal cancer (30), EBV virus LMP2a associated with lymphoma and Hodgkin's disease, hepatitis C virus (HCV) associated with liver cancer, human herpes virus (HHV)-8 associated with Kaposi sarcoma (16), human T cell lymphotropic virus associated with adult T cell leukemia (30). However, a large number of tumor antigens identified so far are not virus associated antigens, but they are actually self-tumor antigens. Based on their tissue expression patterns, expression levels and mutations, these self-tumor antigens can be classified into five groups (16):

Cancer-testis like antigens (CT antigens): CT antigens include MAGE-1, MAGE-2, MAGE-3, MAGE-12, BAGE, GAGE, NY-ESO-1, and CML66 (45), and CML28 (44). Cancer-testis antigen mRNAs are expressed in a wide range of different cancers as well as normal testis (64), but generally are not expressed in most other normal somatic tissues, except testis (54). Since testis is an immune-privileged site that does not express MHC class I or II molecules (65) and may express FasL to kill attacking lymphocytes (66), these antigens can practically be regarded as tumor-specific and are highly desirable as targets for antigen-specific immunotherapy (9, 41, 54, 67-75);

Differentiation antigens: Differentiation antigens are tyrosinase, TRP-1, TRP-2, gp100, MART-1, and MC1R. Since these differentiation antigens are expressed in differentiation stage-dependent and tissue-specific manners, thus, immunotherapy based on these antigens may not cause any side-effects on the other tissues. However, the disadvantage is that antigen-specific immune responses may be compromised by self-tolerance (59, 67);

Tumor-specific antigens: These group of antigens include Ig idiotype, CDK4, caspase-8, β-catenin, Bcr-Abl (68-71), mutated p21/ras, and mutated p53;

Overexpressed antigens: Overexpressed antigens identified are proteinase 3 (myeloblastin) (69, 72), WT-1, MUC-1, normal p53, Her/neu, PAP, PSA, PSMA, and G250;

Oncofoetal antigens: The last group of antigens include CEA, α-fetoprotein, 5T4, onco-trophoblast, and solid tumor-associated glycoprotein. Of note, most of these self-tumor proteins are non-mutated proteins (51).

The hybridoma technology (73) and the recent success of antibody-based immunotherapy (15), such as Rituximab (anti-CD20) and immunotoxins (28, 29) which targets the tumor antigens expressed on the cell surface refresh people with another out-of-date concept (74). This out-of-date concept was that tumor antigens have to be expressed on the tumor cell surface in order to let host immune system to recognize and become tumor antigens. Therefore, the new concept is that although some tumor antigens are expressed in the tumor cell surface (74), most of tumor antigens identified so far, as above-mentioned, are intracellular proteins (7, 44-47).

Are there any principles governing these tumor antigens?

Based on how the genetic information encoding these

self-tumor antigens is organized, we can divide them into two major categories, or groups. The first group comprises *conventional antigens*, such as proteins encoded by genes with conventional exon-intron organization and translated by primary open reading frame (ORF) (55). The conventional tumor-associated antigens include the five groups of tumor antigens above-mentioned (75, 76). The second group comprises *unconventional* cryptic peptide antigens, including cryptic antigens encoded in a) the introns of genes (MPD associated antigen MPD5) (48, 77), b) the exon-intron junctional regions, c) the alternative reading frames (tumor antigen TRP-1) (78, 79) as opposed to the primary reading frames in mRNAs (80-82), d) the subdominant open reading frames located in the 5'-untranslated region (UTR) or 3'UTR of the primary open reading frame (55, 77), and enhanced by chromosome rearrangement, and aberrant processing (30). Recently, we used SEREX technique to screen a human testis cDNA library with sera from three polycythemia vera (PV, a myeloproliferative disease, MPD) patients who responded to interferon-α (IFN-α) and identified a novel unconventional antigens, MPD5. MPD5 belongs to the group of unconventional cryptic antigens without conventional genomic intron/exon structure. MPD5 antigen elicited IgG antibody responses in a subset of polycythemia vera patients, as well as some patients with chronic myelogenous leukemia or prostate cancer, suggesting that it is broadly immunogenic. Upregulated expression of MPD5 in the granulocytes from PV patients after IFN-α (83) or other therapies, might enhance its abilities in elicitation of immune responses in patients. These findings provide new insights into the mechanism underlying the regulation of the self-antigen repertoire in eliciting anti-tumor immune responses in patients with myeloproliferative diseases, and suggest their potential as the target of novel immunotherapy.

What is the significance of identification of unconventional tumor antigens for future immunotherapy? Since these unconventional antigen peptides are not expressed in normal cells and are not tolerated by host immune system, thus, they are considered to be tumor-specific. These features indicate that these unconventional antigens may be desirable to be targets for future immunotherapy (55).

In addition, there are ten other new concepts or principles regarding tumor antigens:

1) A cancer patient can develop immune responses to multiple antigens;

2) A single cancer antigen contains epitopes that can be presented on many different surface HLA molecules. For example, the melanoma antigen gp100 is presented on HLA molecules A2, A3, A24, Cw8, DR4, and DR15, whereas the tyrosinase antigen is presented on A1, A2, A24, B44, DR4, and DR15. Since HLA-A2 and HLA-DR4 are common HLA alleles that are expressed in high percentage of the population (51), more frequently T cell antigen epitopes from tumor antigens restricted by these HLA alleles are identified;

3) Since the processing of HLA class I-restricted tumor antigen epitope utilizes the ubiquitination-proteasome protein degradation pathway (84, 85) and non-proteasome pathway (86, 87), thus, no tumor antigens can escape from its epitope presentation to the HLA class I pathway. Theoretically, every SEREX tumor antigen encodes T cell antigen epitope despite the potential variations in the HLA presenting alleles and the differences in their immunodominance [HLA binding affinity (88) and TAP binding affinity (89, 90)] among epitopes;

4) HLA-restricted T cell antigen epitopes can be promiscuous. Some promiscuous epitopes can be presented by several HLA alleles. For example, MAGE-A3 epitope 146-160 can be presented by HLA-DR7 and HLA-DR4 (91). In addition, Nakatsura et al. showed that antibody binding epitope can be overlapped with HLA-restricted T cell antigen epitope (92). These epitopes are desirable for more effective immunotherapy;

5) Studies of the immune reactivity of patients with CML can lead to identification of antigens expressed and immunogenic not only in CML, but also broadly in other tumors, such as tumor antigens CML66L and CML28 (30, 44, 45);

6) Due to immune self-tolerance, it is not often that the scale of anti-tumor immune responses in patients (44, 45) reaches as high as that of anti-virus immune responses;

7) The tumor heterogeneity develops because the genome of cancer cell is inherently unstable, consequently, altering the cells' genotype and antigen profile (93). Patients with the same type of tumor may have differences in antigen profile. Therefore, detection rates of immune responses to any given tumor antigens in certain patient population are in the range of less than 100%, for example, previous studies showed that the incidence of IgG antibody immune responses to the aberrantly expressed self-tumor antigens ranges between 5% and 50%, depending on the tumor type and the respective antigen (1);

8) Anti-tumor immune responses in most cases are closely related to the special forms of autoimmune reactions. Several reports support this argument. A significant proportion of the currently defined SEREX antigens are also autoantigens (7). Cytogenetic response in patients with CML from Philadelphia chromosome$^+$ to Philadelphia chromosome$^-$ in response to IFN-α therapy is often associated with therapy-related autoimmunity (94). Induction of anti-tumor immunity with self-antigen vaccination eradiates cancer (melanoma) cells, but at the same time causes normal tissue destruction, as manifested by the development of vitiligo (51, 95). Removal of $CD4^+CD25^+$ regulatory T cells (Treg cells) results in autoimmune diseases (2) and enhancement of anti-tumor immune responses by circumventing T cell tolerance (96, 97);

9) Tumor antigens do not have to be proteins, they can be carbohydrate antigens (49). Although carbohydrate antigens are not intrinsic T cell dependent antigens, novel approaches are needed to drive T cell response to these antigens if using carbohydrate antigens as active immunotherapy for tumors (49);

10) The association of antigen-specific immune responses with tumor remission is informative. As we demonstrated previously, high tittered IgG antibody responses to tumor antigens CML66L and CML28 are associated with CML remission induced by donor lymphocyte infusion, suggesting

that antigen-specific immune responses may play an important role in leading tumor remission (44, 45). There is no need to prove that the tumor antigen of interest is really a tumor rejection antigen before to start antigen-specific immunotherapy.

What are the mechanisms for immunogenicity of tumor antigens?

In addition to the immunogenicity underlying some tumor-specific antigens generated *via* mutations (p53 and ras), chromosome translocations and abnormalities, such as expression of Bcr-Abl (68-71), the mechanism underlying the immunogenicity of most non-mutated self-tumor antigens is their aberrantly overexpression in tumors. Zinkernagel et al. suggested that the overexpression of self-antigens or novel antigenic structure of autoantigens (98), overcomes the threshold of antigen concentration at which an immune response is initiated (99). This threshold might be lower for certain untolerized regions of certain antigen epitopes. Overexpressed genes, up to 100 folds, often encode tumor antigens identified by SEREX, which may reflect the inherent methodological bias for the detection of abundant transcript (1).

The overexpression of tumor antigens in tumors can result from transcriptional and post-transcriptional mechanisms. We recently demonstrated that overexpression of tumor antigen CML66L in leukemia cells and tumor cells *via* alternative splicing and alternative promoter is the mechanism for its immunogenicity in patients with tumors, which not only illustrated the overexpression of tumor antigen as a principle but also elucidated its molecular mechanism (48). In addition, cancer-testis antigen expression in tumors has been ascribed to abnormal demethylation (100, 101). Furthermore, since a significant proportion of the SEREX defined antigens are autoantigens (7), for example, CML28 is also an autoantigen Rrp46p (44). We recently demonstrated that a novel mechanism of noncanonical alternative splicing provides the structure basis for expression of untolerized autoantigen transcripts associated with various autoimmune diseases (102) and tumors (103). Several other studies also suggest that alternative splicing regulates the immunogenicity of tumor antigens (7).

Do hematologic malignancies express specific tumor antigens?

From the following examples, you may easily conclude that similar to other types of tumors, hematologic malignancies are not the exception that many tumor antigens have been identified in patients with hematologic malignancies.

Leukemia: Infusion of donor lymphocytes without additional therapy can successfully re-induce remission in 75-80% of patients with relapsed CML after allogeneic BMT, which clearly demonstrated the graft-versus-leukemia (GVL) immune responses. However, the antigens mediate the GVL effect remained undefined (104, 105). Wu in Ritz's team previously found that: 1) in the patients with CML who responded to donor lymphocyte infusion (DLI) therapy, there was an association between a decrease in Ph^+ cells from 100% to 0%, and an increase in the number of peripheral B cells from 9-11% to 40-42%, suggesting augmented antibody production in the CML remission; 2) the serum collected after DLI reacted with more antigens in the cell lysates of CML cell K562 on Western blots, when compared with serum from the same patient collected before DLI. Further studies of these antigens revealed that they are not transplantation-related antigens. Such findings suggest that a potent humoral anti-leukemia immune response associated with CML remission developed after DLI. These results, along with observations on the high rate of CML remission in response to DLI with $CD4^+$ T cells (56), strongly suggest that integrated humoral and T cell immune responses may play a decisive role in CML remission (11). An expression cDNA library was constructed from CML cells collected from three CML patients. This library was screened with sera from the same DLI-responding CML patients using the SEREX technique (11). We have identified 13 CML-associated tumor antigens in this screening in Ritz's lab at Harvard Medical School (46). These 13 antigens include 11 known proteins and two novel proteins. The 11 known proteins are T54, phorbolin 1-related, CHD, 7-60, KIAA0530, RBP-Jk, thymosin-b4, defensin-1, ANG2, RBBP-5, and RAFTK; it is noteworthy that BCR-ABL and proteinase 3 were not identified in this screening. Our results suggest that these antigens are associated with an enhanced immune response leading to CML remission, but they are not associated with natural autoantibodies (106) or antibodies against allo-antigens mediating graft-versus-host disease (GVHD). Further, our findings clearly show that immune responses leading to CML remission are separable from those transplantation-related antigens involved in GVHD. Interestingly, sera collected from four IFN-α therapy responders recognized 9 of 13 CML antigens, suggesting that immune responses against CML in DLI responders and IFN-α responders are, to a certain degree, identical.

In addition, several other leukemia related tumor antigens have been studied: a) myeloid differentiation antigens, such as proteinase 3, neutraphil elastase, myeloperoxidase, cathepsin G (107); b) CML-specific Bcr-Abl antigens, p210-b3a2, p210-b2a2, and p190-ela2 peptides (33, 68-71); c) AML specific tumor antigen, PML-RARα (14) and 17 SEREX antigens associated with AML (108); d) allelic single nucleotide polymorphism (SNP) (109), presented in HLA phenotypes, results in minor histocompatibility antigens (mHA) (54, 110, 111); e) tumor-specific antigens: the Wilms tumor antigen (WT1) (33), survivin and MUC1 (14); f) other antigens including RHAMM (112), SPAN-Xb, MPPP11 (113), 43 MLAA antigens (associated with acute monocytic leukemia) (114), 14 KW antigens (associated with chronic lymphocytic leukemia) (115), and T cell leukemia-associated tumor antigen HUB1 (116) have been identified by SEREX (112, 114, 115, 117, 118), and are currently being characterized.

Lymphoma: Some hematologic tumors represent a unique situation not shared by most solid tumors. For example, B cells can express unique idiotypes resulting from the gene rearrangements involved in antibody production. Because each B cell clone gives rise to a lymphoma uniquely expressing this idiotype, it can serve as a tumor antigen. Vaccination with idiotype antigens in patients with follicular lymphoma, or multiple myeloma successfully has led to objective responses (17). In addition, Liggins et al. identified a number of SEREX antigens associated with diffuse large B-cell lymphoma (119). Moreover, numerous SEREX antigens have been associated with various types of lymphomas including APOBEC3B (mantle cell lymphoma) (120), ATF-2 (Burkitt's lymphoma) (121), GBP-5 splicing variants (122) (cutaneous T cell lymphoma), 9 HD-CL antigens (123) (cutaneous T cell lymphoma), SCP-1 and cTAGE-1 (cutaneous T cell lymphoma) (124).

Multiple Myeloma (MM): Several tumor antigens have been associated with multiple myeloma including hTERT, CYP1B1 (125), BCMA (126) and other 13 MM associated antigens (127).

Myeloproliferative diseases (MPD): Recently we have identified a few MPD associated tumor antigens by screening human testis cDNA library with the sera from patients with PV who responded to IFN-α therapy. This is the first report that has demonstrated that anti-tumor immune responses contributed to PV remission induced by IFN-α therapy (77, 128).

What mechanisms complicate anti-tumor immune response and limit tumor regression?

Tumor immunosurveillance theory suggests that tumors can be recognized and eliminated as a result of natural anti-tumor immune responses that develop in the host (129-132). This argument is supported by the discoveries that: a) the immune system can protect the host against the development of spontaneous and chemically induced tumors; b) the immunogenicity of a tumor is imprinted on the tumor by the immunological environment; c) individuals with tumor sometimes develop spontaneous reactivity against the antigens of the tumor (129-132). However, many influences either from tumor or environment render a tumor either invisible to the host immune system or resistant to the anti-tumor immune responses. Several situations can lead to this result: a) the tumor is non-immunogenic, either because it never expressed any tumor antigens or lost them during tumor development, or the tumor acquired defects in the capacity to present tumor antigens to immune cells; b) the immune system may not be able to recognize or eliminate a tumor because the tumor produces immunosuppressive moieties and induces immunosuppressive responses (also see several excellent reviews) (129-133). Due to this complicated situation, antigen-specific immunotherapy may not be able to be effective alone in eradiating tumors. Therefore, it takes two to tango. Future immunotherapy could be in a combinational format including both antigen-specific immunotherapies and anti-tumor immune enhancement therapies.

Concluding comments

If we type "tumor antigen" as a key words in August 2005, we could easily find more than 60,000 publications and 6,300 reviews listed in the PubMed database. Therefore, studies in identification of tumor antigens and antigen-specific immunotherapies have made significant progress and have entered the mainstream of current immunological research and cancer research (4, 30, 35, 52, 134). What do we expect from studies on tumor immunology and immunotherapy? Why do we need to characterize so many tumor antigens? Tumor heterogeneity develops because the genome of cancer cells is inherently unstable. Stoler et al. estimated that about 11,000 genomic alternations occur in a cancer cell (135). In cancer cells, errors in DNA replication can go unrecognized. Such random genetic errors can persist, altering the cells' genotype and antigen profile (93). These features of tumor cells determine the formats of future immunotherapies: a) multi-formats such as peptide vaccination, DNA vaccination (136); b) poly-valents (epitopes) including multiple antigen epitopes; c) combinational approaches with other cytokines, such as IL-2 (30), IFN-α, IFN-γ, dendritic cells, CD40-activated B cells (137), immune-enhancing adjuvants, circumventing T cell tolerance to tumors by depletion of $CD4^+CD25^+$ regulatory T cells (138); d) patient-tailored antigen-specific approach in combination with universal antigen-specific approach (139). The complementary approaches outlined here would lead to the future development of more effective, less side-effect antigen-specific immunotherapies.

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