Tumour Antigens Recognized by Antibodies

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Antigens are frequently overexpressed on tumours, and the recognition of these antigens by antibodies can be used for tumour characterization, and for potential diagnostic and therapeutic purposes.

Secondary article

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Introduction

Considerable progress has been made in the last few decades in the identification of defined tumour-associated antigens recognized by monoclonal antibodies (mAbs). The generation of mAbs against tumour antigens has principally been through standard hybridoma techniques following immunization of mice, while in the last decade recombinant phage display technology has also been employed.

Much of the process of identification and characterization of tumour antigens recognized by mAbs has involved the careful serological analysis and rigorous immunohistochemical screening of normal tissues and tumours. These methods differ from the genetic approach typically used to identify antigens recognized by cytotoxic T lymphocytes (CTLs), and the molecular definition of many of these antigens is therefore less well defined. Similarly, the function of many tumour-associated antigens (especially cell surface antigens in solid tumours) is often poorly understood. The importance of these tumour antigens is that they can be utilized for diagnostic and potential therapeutic purposes, and can also provide prognostic information for patients with cancer. The use of serum mAbs to identify tumour antigens through recombinant tumour complementary deoxyribonucleic acid (cDNA) libraries (SEREX) has recently emerged as a powerful new method for the characterization of novel tumour antigens, and the methodology, results and implications of this technique are also discussed in this article.

Antigens Overexpressed in Tumours

Most antigens in tumours recognized by mAbs are encoded by nonmutant cellular genes that are expressed not only by certain cancer cells, but also by at least one subset of normal adult cells. Therefore, these antigens are not tumour specific, but are often referred to as tumour-associated antigens. Many of these antigens are expressed at least to some degree on the cell type from which the tumour developed, and as such are lineage specific and represent differentiation antigens. The normal tissue distribution of these antigens may be quite extensive (e.g. gangliosides, growth factor receptors); however, tumours often express these antigens at higher levels than normal tissues (often up to 100-fold), and the accessibility of antigen on tumours to circulating mAbs may be greater than in normal tissue. These antigens represent a very diverse group of proteins, glycoproteins (including mucins) and glycolipids. The expression of antigens recognized by mAbs in tumours is often heterogeneous, and loss of expression may be observed in anaplastic transformation and as a result of immune escape.

The classification of tumour antigens recognized by mAbs is presented according to antigen structure, and the component of tumours expressing the antigen, in view of the similarities in genetic and functional characteristics of these groupings.

Haematopoietic Differentiation Antigens

Leukaemias and lymphomas are characterized by malignant cells that express immunophenotypes that are found in selected stages of haematopoietic differentiation. These antigens are usually associated with cluster differentiation (CD) groupings and have a glycoprotein structure, but are not always strictly tumour or lineage specific. The ideal antigen target for mAbs directed against leukaemias or lymphomas has a high expression in neoplastic cells, but not on normal cells or bone marrow progenitors.

The antigens most commonly identified by mAbs in leukaemia and lymphoma, and which are potential targets for diagnosis and therapy, are detailed in **Table 1**. For leukaemias, antigen targets include CD33 (acute myeloblastic leukaemia, chronic myelocytic leukaemia) and CD45; CD5 is expressed in T-cell leukaemias/lymphomas; nonHodgkin lymphoma antigen targets include CD19, CD20, CD21, CD25 and CD37. The success of targeting

Table 1 Tumour antigens recognized by antibodies

Antigen category	Antigen name	Tumour types
Haematopoietic differentiation antigens	CD5	T-cell leukaemia/lymphoma
	CD19, CD20, CD21, CD25, CD37	B-cell lymphoma
	CD30	Hodgkin lymphoma
	CD33, CD45	Acute myeloblastic leukaemia
	CAMPATH-1 (CDw52)	Lymphoid malignancies (T and B cell)
	HLA-DR	B-cell lymphoma
	Anti-idiotype	B-cell malignancies
Glycoproteins	Carcinoembryonic antigen (CEA)	Epithelial tumours (breast, colon, lung)
	TAG-72, Ep-CAM, MUC1	Epithelial tumours (breast, colon, lung)
	Folate-binding protein	Ovarian tumours
	A33	Colorectal carcinoma
	G250	Renal carcinoma
	Prostate-specific membrane antigen (PSMA), Prostate specific antigen (PSA)	Prostate carcinoma
	Ferritin	Hodgkin disease, hepatoma
Glycolipids	Gangliosides (e.g. GD2, GD3, GM2)	Neuroectodermal tumours, some epithelial tumours
Carbohydrates	Le^y	Epithelial tumours (breast, colon, lung, prostate)
	CA-125	Ovarian carcinoma
	CA19-9	Epithelial tumours
Growth factor receptors	Epidermal growth factor receptor (EGFR)	Lung, glioma, breast, head and neck tumours
	$p185^{HER2}$	Breast, ovarian tumours
	IL-2 receptor	T- and B-cell neoplasms
Mutated gene products	de2-7 EGFR	Glioblastoma multiforme, breast, lung cancer
Stromal or vascular antigens	Fibroblast activation protein (FAP)	Epithelial tumours (colon, breast, lung, head and neck)
	Tenascin, metalloproteinases	Glioblastoma multiforme, epithelial tumours
	Endosialin, Vascular endothelial growth factor (VEGF), $\alpha_V\beta_3$	Tumour vasculature

CD20 in the clinic has been demonstrated by the recent approval in the USA and Europe of an anti-CD20 mAb for the treatment of nonHodgkin lymphoma. Other targets include the CDw52 antigen, which is expressed in mature lymphoid malignancies, and CD30 in Hodgkin lymphoma. The accessibility of leukaemia and lymphoma cells to circulating mAbs provides distinct advantages in antigen targeting compared with solid tumours.

Cell Surface Differentiation Antigens

Glycoproteins

Glycoprotein antigens expressed on the cell surface of solid tumours are common targets for monoclonal antibodies. These antigens have varied expression patterns in normal tissue, ranging from extensive tissue distribution to restricted expression, and are found predominantly on tumours of epithelial origin. The carcinoembryonic antigen (CEA), sialyl Tn (tumour-associated glycoprotein (TAG-72)) antigen, and the *MUC1* gene product polymorphic epithelial mucin (PEM) have been the tumour antigens most studied in animal models and clinical trials with mAbs. The CEA antigen is expressed on a number of tumours of gastrointestinal origin, and in other tumours such as breast and lung cancer. Although originally described as an oncofetal antigen, CEA is in fact also expressed in nonmalignant, nonfetal adult tissues such as normal colonic mucosa, lung and lactating breast tissue. CEA is shed from the surface of tumour cells, and raised serum levels of CEA have therefore been used to detect occult tumour; however, increased serum levels may also

be found in smokers and in patients with inflammatory bowel disease.

All these antigens have variable expression in a broad range of epithelial tumours, and soluble forms of these antigens may be found in the serum of patients with these tumours. Both PEM and epithelial cell adhesion molecule (Ep-CAM; a 40-kDa glycoprotein targeted by the mAb 17-1A and involved in cell adhesion) are expressed in epithelial tumours and in a wide range of normal epithelial tissues. In most tumours that express these antigens, downregulation of antigen in metastases compared with activity in primary tumours has been reported.

Glycoprotein antigens with distinct, high tumour expression and restricted normal tissue distribution include A33 and G250. The A33 antigen is a 43-kDa protein which has restricted expression in human small and large intestine epithelial cells, and is found in more than 95% of human colorectal carcinoma cells. Upon mAb binding to A33 antigen, the antibody–antigen complex is internalized and sequestered in vesicles. The unique structure of the A33 antigen has been shown to consist of a transmembrane glycoprotein with an extracellular domain structure related to the CD2 subgroup of the immunoglobulin superfamily. The function of this antigen is as yet undefined, but it may be a novel cell surface receptor or involved in cell–cell adhesion. Another glycoprotein antigen with restricted normal tissue expression is G250, which is expressed by more than 90% of clear cell renal carcinomas but not in normal kidney, and is also expressed in biliary epithelium. The G250 antigen has been shown to be a transmembrane protein identical to the tumour-associated antigen MN/CA9. mAbs directed against the A33 and G250 antigens have been studied in clinical trials, and have demonstrated excellent targeting characteristics indicating the suitability of these antigens for directed cancer therapy.

Glycolipids

Gangliosides are glycolipids expressed in animal cells, and consist of a carbohydrate moiety linked to a ceramide molecule which is anchored in the lipid bilayer of the cell wall. Malignant transformation of cells may be associated with qualitative and quantitative changes in ganglioside expression on the malignant cell surface, which can be recognized immunologically. Gangliosides are known to be immunosuppressive cell surface molecules and may be shed actively by tumour cells. Ganglioside synthesis may be incomplete and result in precursor accumulation (e.g. GD2, GD3, GM2, GM3, which are found on tumours of neuroectodermal origin – (melanoma, neuroblastoma, astrocytoma and small cell lung carcinoma). Modifications to the carbohydrate or ceramide portions may also be observed. Expression of gangliosides in normal tissues is often widespread (especially GM2 and GD3). The gangliosides GD3, GD2 and GM2, in particular, are expressed on the cell surface of malignant melanomas, GD3 being the most abundant, and expression of GD3 is linked to the loss of differentiation in premalignant naevi as they progress to malignant melanoma. The high expression of some gangliosides in tumour cell membranes may also lead to conformational changes of antigen epitopes which enhance monoclonal antibody binding. The function of gangliosides is believed to involve cell growth and differentiation, and gangliosides may also have functional roles in attachment to extracellular matrix proteins and thereby enhance the metastatic potency of tumour cells expressing gangliosides on the cell surface.

Carbohydrates

A range of carbohydrate determinants linked to either lipids or proteins has been identified by serological analysis of human cancer cells with mouse mAbs. Blood group-related antigens (e.g. Le^b and Le^y) have been the focus of much attention because of their high level of expression in epithelial tumours, although some expression in normal tissues is also present. The antigens sialyl Le^a and sialyl Le^x are frequently expressed on human cancer cells and serve as ligands for a cell adhesion molecule of the selectin family, E-selectin, which is expressed on vascular endothelial cells. Sialyl Le^a and sialyl Le^x are also involved in the adhesion of cancer cells to vascular endothelium and thus contribute to haematogenous metastasis of cancer. Another antigen, Le^y, is expressed in more than 70% of epithelial cancers (including breast, colon, ovary and lung cancer) and is an attractive target for mAb-directed therapy (**Figure 1**). A number of mouse mAbs against Le^y antigen have been produced, although a consistent problem with these mAbs has been an element of cross-reactivity with Le^x and H type 2 structures, and agglutination of human red blood cells;

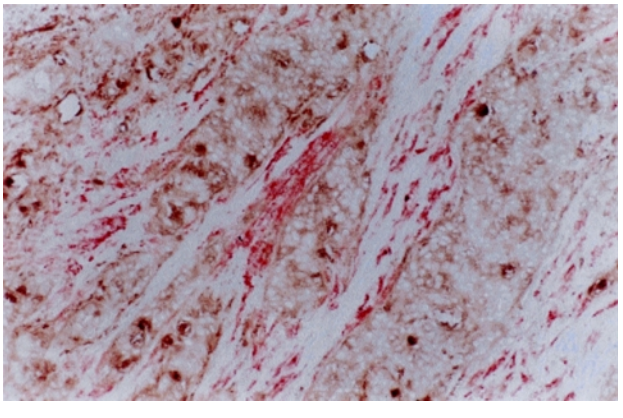

Figure 1 Immunohistochemical detection of tumour cell surface and stromal antigens in breast cancer. The red staining corresponds to human fibroblast activation protein (FAP) expression on fibroblasts in the stroma of the tumour. The brown staining corresponds to Le^y antigen expression on the cell surface of the breast cancer cells.

meticulous serological analysis has been required to identify anti-Le^y mAbs that have minimal or no cross-reactivity with other blood group-related antigens. Le^y antigen expression has been shown to be associated with more differentiated tumour and with a significantly improved patient prognosis in nonsmall cell lung cancer.

Growth Factor Receptors

Growth factors may be overexpressed in tumours, and the growth or survival of some tumours may partially depend on autocrine or paracrine production of epidermal growth factor (EGF) or transforming growth factor α (TGFα). These two factors share a common receptor, the EGF receptor (EGFR), which is expressed in normal tissue (particularly the skin and liver), but has increased expression in certain tumours (e.g. lung and breast). The antitumour activity of most EGFR antibodies is enhanced by their ability to block ligand binding, although such antibodies may mediate their efficacy through both modulation of cellular proliferation and antibody-dependent immune functions (e.g. complement activation). A truncated mutant EGFR (de2-7 EGFR or EGFRvIII), which contains a deletion of 267 amino acids from the extracellular domain and does not bind EGF, has also been identified. In adults, the expression of the de2-7 EGFR appears to be restricted to tumours exhibiting amplification of the EGFR gene. While this truncated receptor does not bind ligand, it does possess low constitutive activity and imparts a significant growth advantage to glioma cells; it is associated with increased proliferation and reduced apoptosis. mAbs against the de2-7 EGFR have been generated, and may represent an important new therapeutic strategy for glioma and other epithelial tumours.

Another important receptor is the transmembrane glycoprotein receptor $p185^{HER2}$ (encoded by the human epidermal growth factor receptor 2 (HER-2)/neu oncogene). $p185^{HER2}$ has partial homology with the EGFR, is overexpressed in 25–30% of breast cancers, and is associated with a worse prognosis in patients with primary disease involving axillary lymph nodes. mAbs directed against $p185^{HER2}$ have been shown to inhibit the growth of tumours in animal models, and of transformed cells expressing high amounts of this receptor. Objective responses in patients with metastatic breast carcinoma overexpressing $p185^{HER2}$ with mAbs directed against the extracellular domain have been reported, and recent approval of antibody therapy directed against this receptor in patients with breast cancer indicates the potential therapeutic uses of growth factor-targeted therapy.

Angiogenesis and Stromal Antigens

The extracellular matrix has a critical role in the formation of tumours, both because of its role as a supporting structure for tumour growth, and the production of factors and enzymes that are involved in angiogenesis and tumour cell migration. The human fibroblast activation protein (FAP) is a cell surface antigen expressed in reactive stromal fibroblasts of a number of epithelial cancers including colon, breast and lung (**Figure 1**). FAP is absent or expressed in low levels in most adult tissues, but is seen in wound healing and transiently in some fetal mesenchymal tissues. FAP has structural homology to CD26, but appears to be functionally distinct, and FAP has both dipeptidyl peptidase activity and a collagenolytic activity capable of degrading gelatin and type I collagen. The successful targeting of mAbs against FAP expressed in tumour stroma has been demonstrated in clinical trials of patients with colorectal carcinoma. Another potential stromal antigen target is tenascin, an extracellular matrix glycoprotein present in developing tissue during the fetal period and expressed in the stroma of many adult tumours, particularly glioblastoma multiforme.

Serological studies have also identified a number of antigens expressed selectively on neovascular endothelium of tumours, including endosialin, vascular endothelial growth factor receptor (VEGFR) and the integrin $\alpha_V\beta_3$. VEGF is an important factor in tumour angiogenesis, and neutralizing mAbs to VEGF have shown inhibitory effects on tumour growth in mouse models. VEGF expression has also been linked to poor outcome in patients with cancer including nonsmall cell lung cancer. The restricted expression of these antigens in tumour vasculature, and the potential antitumour effects of inhibitors of these antigens, has been the subject of intense interest as a means of killing tumour cells without requiring specific tumour cell targeting.

Applications of Antibodies to Tumour Antigens

The clinical applications of mAbs recognizing tumour-associated antigens include both diagnosis and therapy. Diagnostic uses include serum detection of soluble antigen as a marker of disease activity (e.g. CEA, CA-125 and prostate-specific antigen (PSA)). The use of these markers must always be in the clinical context of the individual patient, however, as there are no immunological serum markers presently available that are specific for cancer, and levels of presently available serological markers may also be raised in a variety of nonmalignant diseases and conditions. Immunohistochemical characterization of biopsy specimens for pathological diagnosis can also be performed with mAbs, although this technique needs to

take account of potential aberrant expression patterns for some antigens, and antigen downregulation with loss of differentiation of tumour. An additional use of radiolabelled mAbs is for tumour detection *in vivo* (e.g. anti-CEA, anti-TAG-72, anti-PSMA (prostate-specific membrane antigen) mAbs). Perhaps the most promising area of clinical development, however, is in mAb-targeted therapy. Based on the experience of clinical trials to date, the ideal therapeutic antigen target has homogeneous expression in tumour, minimal expression in normal tissues, no (or minimal) soluble form of antigen to impede tumour binding, and easy accessibility to mAbs. Tumour antigen screening, and an understanding of the internalization characteristics and function of targeted antigen, permits the optimal selection of mAbs for targeting strategies in the clinic. The range of tumour antigens recognized by mAbs and the expanding therapeutic strategies that can be employed will form the basis of continuing clinical research.

Identification of Tumour Antigens with Autologous Sera

Antibody response to tumour antigens

The search for autologous antibodies recognizing antigens expressed specifically by tumour cells was initiated in the 1960s. At this time, studies were hampered by technical limitations as polyclonal patient serum revealed in most cases a strong reaction with normal tissue antigens, but did not show tumour specificity. The development of the hybridoma technology by Köhler and Milstein raised the hope that monoclonal antibodies could uncover tumour specific antigens in humans. This technique allowed the characterization of a large variety of new antigens that might be used as potential targets for immunotherapeutic approaches. However, only a minority of these antigens seemed to evoke an immune response in patients with cancer and could be used as surrogate markers. To date, the clinical significance of B-cell responses to tumour antigens still remains unknown. For example, while the presence of p53 antibodies is associated with a poor prognosis, the clinical significance of anti-HER-2/neu antibodies has not yet been determined. More patients must be analysed to distinguish whether the development of antibodies to tumour antigens is associated with clinically relevant features or might be used for diagnosis and prognosis.

Cellular immune response to tumour antigens

Clinical observations in patients with cancer who have spontaneously regressing tumours have always supported the hypothesis of an existing antitumour response in a subset of patients. Several tumour entities are now defined where this phenomenon can be observed, with a frequency ranging between 5 and 15%. T lymphocytes have been identified to be the prime component of this antitumour response, as tumour-specific CTL clones could be raised from patients suffering from melanoma, renal cancer and breast cancer. In addition, tumour-specific precursor CTL (pCTL) clones can be detected in a variety of patients with cancer, with an increase in number after antigen-dependent vaccination with either protein, peptide or DNA vaccines. As the induction and expansion of tumour-antigen specific CTL clones has correlated in some patients with a clinical response and tumour regression, tumour immunologists have focused principally on T cells for the detection and characterization of tumour-associated antigens. One approach made use of antigen-loss tumour cell variants transfected with cDNA isolated from tumour tissue and cytotoxic CD8 + antitumour T-cell clones (CTL). The second approach has been based on a biochemical strategy using acid elution of antigenic peptides bound to major histocompatibility complex (MHC) class I molecules from tumour cells. These strategies have helped to define several new human tumour antigens at the molecular level, most notably in malignant melanoma. The difficulty in expanding specific T-cell clones for the majority of tumour entities is a major obstacle for the general application of these strategies.

Characterization of tumour antigens by SEREX (*se*rological analysis of antigens by *r*ecombinant *ex*pression cloning)

To overcome the limitations implemented by the T cell-based methods, a new strategy using autologous serum for the characterization of tumour antigens has recently been described. This novel technique, called SEREX, allows for the direct molecular definition of new tumour antigens that elicit an immunoglobulin G antibody response in patients with a tumour. By screening procaryotically expressed tumour-derived cDNA libraries with autologous sera, several new antigens in different tumour entities have been identified, including the MAGE-1 (melanoma tumour antigen) and tyrosinase antigens, which have been originally defined by their T-cell reactivity. **Table 2** lists the categories of tumour antigens that have been identified to date.

One of the first antigens characterized by SEREX, a renal specific carbonic anhydrase, provided proof of the long-standing concept that nonmutated cellular antigens that are amplified or overexpressed can serve as immunogenic antigens. Several new antigens discovered recently (galectin 9, aldolase A and translation initiation factor eIF-4γ) have confirmed this observation. The most fascinating group of antigens is the cancer–testis (CT) antigen group. These antigens are expressed by a variable proportion of

Table 2 Categories of SEREX identified tumour antigens

Antigen category	Antigen name	Tissue source
Amplified or overexpressed	Carbonic anhydrase	Renal cancer
	Galectin 9	Hodgkin disease
	Aldolase A	Lung cancer
	eIFγ4	Lung cancer
Differentiation	Tyrosinase	Melanoma
	Galectin 4	Colonic cancer
Retroviral	HERKV-K10	Renal cancer
Mutational	p53	Colonic cancer
Chromosome 3p	NY-LU-12	Lung cancer
Splice variant	Restin	Hodgkin disease
	NY-CO-38	Colonic cancer
Cancer testis (CT)	MAGE-1	Melanoma
	MAGE-4a	Melanoma
	SSX2	Melanoma
	NY-ESO-1	Oesophageal cancer
	SCP-1	Normal testis

Classification of antigens has been performed according to their genomic characteristics. Note: The SCP-1 antigen was identified by screening a pooled testis library from healthy donors, which was subtracted against normal tissues with serum from a patient with renal cancer.

different tumour types but are highly restricted in their expression pattern in normal tissues, with testis being the sole or predominant site. Three antigens in this category, MAGE, BAGE (breast tumour antigen) and RAGE (renal tumour antigen) were initially identified as targets for cytotoxic T cells. A variety of new CT antigens (HOM-MEL-40, NY-ESO-1, SCP1) have now been uncovered by SEREX analysis. As no mutation, rearrangement or amplification has been observed for the genes encoding these CT antigens, the most likely explanation for their expression as tumour antigens is gene activation or derepression.

The high titre of autologous tumour-specific antibodies detected by SEREX implies that cognate CD4 + T helper (T_H) cell immunity might be present and operative in antibody-positive patients. The impact of CD4 T_H cells on an antigen-specific tumour response has not been studied extensively to date, as most researchers have concentrated on CD8 + cytotoxic T cells. However, evidence has accumulated that CD4 + T cells play a critical role in the antitumour response by mediating critical priming and effector functions. For example, significant antibody titres of 1 : 25 000 and higher have been demonstrated against the NY-ESO-1 antigen in patients with melanoma, supporting the existence of a CD4 + T cell-mediated B-cell expansion. The coexistence of CD4 + T cells and a B-cell response have also been clearly demonstrated for the HER-2/neu protein in patients with breast cancer. In addition, it has been shown that CD4 + T-cell lines and clones cultured from tumour-infiltrating lymphocytes recognize epitopes that were products of the same tyrosinase gene that was shown to encode class I-restricted peptides recognized by CD8 + T cells. These results, together with the observation that classical tumour antigens defined by T-cell responses such as MAGE-1 and tyrosinase can also be detected by the serological approach, suggest that an integrated immune response against tumour antigens may exist that involves both CD8 + and CD4 + T cells as well as B cells. Further evidence of this integrated response was observed in a recent NY-ESO-1-based vaccine trial, where a high proportion of patients with NY-ESO-1 antibody also had detectable CD8 + T-cell responses to known human leucocyte antigen (HLA)-A2-restricted NY-ESO-1 peptides. Indeed, antibody and CD8 + T-cell responses to NY-ESO-1 occurred only in patients with NY-ESO-1-expressing tumours, and CD8 + T-cell responses to NY-ESO-1 have not been detected in patients without NY-ESO-1 antibody, demonstrating clearly the close link between antibody and cellular immune response at least for this SEREX-defined protein.

As the number of SEREX-defined tumour antigens continues to increase in the near future, the importance of tumour-specific antibody responses in patients with cancer will be further strengthened. Antibody response to new tumour antigens might also lead to the development of new screening methods for the detection of cancers, and will

increase the number of tumour antigens recognized by both the humoral and cellular part of the immune system. Finally, this approach may help to overcome the artificially drawn border between T- and B-cell immunologists in their search for new tumour antigens, and will help to focus on the ultimate goal of optimizing therapy and detection of cancer for the benefit of patients.

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