Review

Tapping the treasure of intracellular oncotargets with immunotherapy

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A B S T R A C T

It is commonly believed that antibodies are too large (\sim 150 \text{ kDa}) to access the intracellular compartment. Therefore, therapeutic antibodies have been traditionally used to target cell surface receptors or soluble proteins in the circulation, leaving a large intracellular treasure of potential cancer-specific targets untapped. This review offers new perspectives on our recently proposed concept that antibodies can be used to target intracellular tumor antigens for anti-cancer therapy. We propose to vastly expand the repertoire of potential targets for cancer immunotherapy since many excellent cancer targets are inside cells.

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1. Introduction

1.1. The global commercial market for therapeutic antibodies

The vision of antibodies as ‘magic bullets’ that could be used to selectively target disease-causing organisms was first proposed by Paul Ehrlich more than a century ago [1]. With the emphasis and recent success on personalized medicine and drug specificity, monoclonal antibodies (mAbs) are one of the largest classes of drugs today and have been very commercially successful. Because antibodies can mark cancer cells to be more visible to the immune system, they are far more specific than small-molecule drugs; they are not inherently toxic and are better tolerated. Antibody-based drugs have achieved remarkable clinical and commercial success. In 2009, the global market for mAbs was $36.4 billion [2]. In 2011, antibodies generated in excess of $45 billion in sales [3]. More than 40% of this revenue came from anticancer mAbs such as rituximab, cetuximab, trastuzumab, and bevacizumab. In the United States alone, the demand for biologics is expected to grow 6.5% a year, while the cancer vaccines market is forecast to grow at a compounded annual rate of 21% [2]. Some analysts predict that with the rate of current revenue growth and potential new approvals, the global market could reach $58 billion by 2016 [3]. This rate of growth would correspond to 40% of projected drug spending in 2020 on specialty drugs.

1.2. Antibody drugs are generally employed to target extracellular oncoproteins

Intracellular oncoproteins were thought to be inaccessible by antibodies, even though there is no evidence with immunotherapy in mice to support this assumption. As a result, many promising intracellular targets have only guided development of small molecule drugs, but not antibody-based immunotherapy. The ‘druggable’ candidates for antibody are perceived to only include the extracellular oncoproteins. Therefore, several current available monoclonal antibody drugs are generated only to target cell-surface receptors or soluble proteins for various types of cancers.

1.3. Antibodies can penetrate live human cells

Since the early 1970s, accumulating evidence has suggested that it is possible for antibodies to access intracellular compartments. Immunologists have demonstrated that antibodies to ribosomal proteins are able to penetrate to intranuclear localization in live human mononuclear cells through Fc receptors-mediated endocytosis [4,5]. Antibodies could also penetrate viable human lymphocytes [6]. Immunogenic activity of antibodies against intracellular antigens have been implicated in apoptotic pathways [7,8] and autoimmune diseases [9,10]. In 1975, it was reported that...
anti-RNA antibodies were taken up by normal (3T3) and simian virus 40-transformed (SV 3T3) mouse fibroblasts. The anti-RNA antibodies retain their anti-antigenic properties and block cellular proliferation in SV 3T3 [6]. Furthermore, anti-DNA antibodies could enter nucleus via caveoli-mediated process and subsequently modulate expression of caveolin and p53 [11]. Recently, tripartite motif-containing 21 (TRIM21) was shown to be able to mediate antibody function inside cells by engaging with a potent effector mechanism that targets the virus for degradation [12].

1.4. Emerging evidence suggests that immunotherapy can be used to target intracellular ‘tumor antigens’ for anti-cancer therapy

In 2008, for the first time, we reported that it is viable to target intracellular ‘tumor antigen’ with antibody therapies for anti-cancer in mice. mAbs could target intracellular PRL (phosphatase of regenerating liver) phosphatases, and thus inhibit experimental metastases [13]. The oncogenic PRL-phosphatases that are hidden at the inner layer of the plasma membrane or endosomes are often overexpressed in cancer cells and associated with metastatic progression of multiple human cancers [14]. Importantly, these PRL-specific antibodies could reduce the metastatic tumor burden that expressed the corresponding intracellular PRL-phosphatase in nude mice [13]. Conversely, tumors targeted with unrelated antibodies produced no beneficial response. Therapeutic efficacy is highly dependent on antibody–antigen specificity. We further explored this finding that proteins hidden within cells can be attacked by antibodies. In 2011, we proposed the important concept of targeting intracellular oncoproteins with antibody therapy or vaccination [15]. Three stable cell lines overexpressing three distinct intracellular antigens (PRL-3, GFP, middle T) were generated. Each of these cancer cell lines was injected via the tail vein into mice to induce experimental metastatic lung tumors. Subsequently, the three corresponding antibodies were injected via the tail vein of mice to inhibit tumor formation. Notably, tumors targeted with unrelated antibodies or tumors without expressing the corresponding intracellular target showed no therapeutic response, suggesting that ‘antibody–antigen’ complexes have to be specific in order to achieve therapeutic efficacy. Particularly, the efficacy seen using a general non-oncogenic reporter (green fluorescent protein) and spontaneous tumor models (polyomavirus middle T oncoprotein) suggests that this is not only an intrinsic property of specific antigens, but a generalized principle that is applicable to a wide variety of intracellular tumor antigens. Vaccination with each of the intracellular proteins stimulated production of specific antibodies by the host immune system which then led to specific tumor regression, resulting in a survival advantage in vaccinated mice. Thus, antibody therapy or vaccination can both be used to target intracellular oncoproteins for cancer treatment. The therapeutic efficacies from both antibody therapy and vaccination are similar. In 2012, we generated the first chimeric antibody targeting the PRL-3 intracellular oncoprotein that was first linked to human colorectal cancer liver metastases by Vogelstein group in 2001 [16]. The PRL-3 chimeric antibodies could inhibit tumors formed by melanoma cell lines expressing endogenous PRL-3 protein [17]. To be more clinically applicable, we further used PRL-3 antibodies to treat FLT3-ITD-associated acute myeloid leukemia (AML) which often induces PRL-3 overexpression. PRL-3 mAb exerted therapeutic effects in mice carrying PRL-3 expressing FLT3-ITD cancer cells [18]. Although antibodies have been used in these mice as mono-therapy, in translating these to the clinical setting, these therapies may potentially also be adjuvant to conventional chemotherapy, radiation, or surgery as well. A study in 2012 reported that antibodies could target intracellular NY-ESO-1, a widely expressed immunogen in human tumors that is expressed intracellularly rather than on the surface of cells [19]. This effect is enhanced when combined with chemotherapy. They showed that intracellular tumor antigens can be captured by mAbs and engaged in an efficient induction of CD8 T-cell responses, suggesting the possible use of mAbs for passive cancer immunotherapy. Furthermore, in 2013, another research group has also reported that targeting the intracellular Wilms tumor 1 (WT1) oncoprotein with human antibodies reveals therapeutic effects in mice [20]. WT1 oncoprotein is an intracellular oncogenic transcription factor overexpressed in a wide range of leukemias and solid tumors. This finding also provides preclinical validation for the strategy of developing therapeutic mAbs targeting intracellular oncogenic proteins. A timeline of these studies is illustrated (Fig. 1) to demonstrate the possibility of immunotherapy targeting intracellular proteins for anti-cancer therapies.

2. Mechanisms of antibodies targeting intracellular antigens

2.1. In vitro mechanisms

There are various hypotheses to explain the mechanisms of antibodies targeting intracellular antigens for anti-cancer effect. In 2008, we showed that antibody could enter live cells via endocytosis which however has no cell-killing effect in culture conditions [13]. This is expected because the in vitro system is not representative of in vivo complex of biological systems. The in vitro system is a single cell type grown in medium supplemented with 10% FBS in an incubator. In contrast, the in vivo system consists of multiple cell types/organs, immune-system, blood circulation system to achieve final cancer cell-killing effect. Therefore, in vitro cell-culture system is unable to mimic in vivo complexity. In 2011, Ferrone elegantly proposed three possible mechanisms in his Perspective [21] on our study for the antitumor activity of intracellular tumor antigen (TA)-specific mAbs. Intracellular TAs might: (i) interact with mAbs that have been internalized (through a B-cell mediated mechanism) by tumor cells, which are then destroyed, (ii) migrate to the cell membrane and bind to mAbs on the surface, and the TA-mAb complex is recognized by Fc receptors on NK cells which carry out antibody-dependent cytotoxicity, or (iii) be shed into the microenvironment or circulation to form complexes with circulating mAbs. We hypothesized that these TAs are shed into blood circulation due to cell lysis or unconventional secretion [22]. Although soluble tumor antigens may potentially neutralize the therapeutic effect of antibodies, the complex could then be taken up by dendritic cells, which process the TAs and present the resultant TA peptides to cognate T cells. Activated T cells then mediate tumor-cell killing. Cancer antigens (regardless extra- or intra-cellular location) are generally more concentrated near the cancer mass due to continuous shedding; the antigen–antibody complexes may induce more localized immune responses to the cancer cells by recruiting lymphocytes and other components of the immune system. This could be part of the mechanism underlying the therapeutic effects of antibodies against intracellular oncoproteins.

2.2. In vivo mechanisms

We investigated possible in vivo mechanisms by scoring immunotherapeutic efficacies based on our studies [13,15,17,18] (Fig. 1, labelled with ‘) using more than 4000 mice from 8 different mouse strains (Table 1) and identified several key factors that could contribute to therapeutic efficacy. (1) The Antigen-antibody specific interaction is critical for therapeutic effect. Tumors targeted with unrelated antibodies produced no beneficial response, suggesting that the antibody is dependent on its specific antigen for efficacy. (2) B-cells & NK cells (but not T-cells) are required for therapeutic efficacy. We showed therapeutic efficacy in a nude mouse model.
(T-cell deficient), but not in a Severe Combined Immunodeficiency' (Scid) mouse model (B- and T-cell deficient). Furthermore, therapeutic effects were seen in C57BL6 wild type but not in muMT mice (B-cell deficient). Both Scid mice and muMT mice have no B-cells and do not show therapeutic effects [15,23]. (3) While the full Fc fragment is essential, subtypes of antibodies are not critical. We used several different subtypes of antibodies (IgG1, IgG2a, IgG2b, rabbit antibodies) to target intracellular ‘tumor specific’ antigens. The therapeutic responses are highly dependent on the expression levels of the respective targets, but independent on subtypes of the antibodies. Furthermore, when we used PRL-3 mini-body where the CH1 and CH2 domains are absent from Fc fragment, we observed little therapeutic effect, suggesting that the complete Fc fragment is essential for antibody response (unpublished data). (4) Complement 5 may not be involved. We next used MMTV-PymT transgenic mice that are commonly used as excellent spontaneous tumor models for decades by the cancer research community. MMTV-PymT transgenic mice carry the mT intracellular DNA viral oncoprotein under the transcriptional control of the mouse mammary tumor virus promoter/enhancer as a model of oncogene-induced mammary tumorigenesis [24]. The mT expression is detected at high levels in mammary glands, and the expression of mT oncogene is sufficient for mammary epithelial cell transformation [25]. All untreated female carriers (genotype +/-) develop palpable mammary adenocarcinomas at the age of 2–3 months. Untreated mice carried marked breast tumors and multiple lung tumors, whereas 83.4% (15/18) of mT antibody-treated mice showed marked reduction in the formation of metastatic breast tumors when examined at 12–13 weeks [15]. These results suggest that the extensive repression of spontaneous tumor formation could be achieved by treating MMTV-PymT mice with mT antibody alone. MMTV-PymT mice were derived from the parental FVB/Nj background, which fail to secrete complement 5 due to a 2-bp deletion in the Hc gene, causing a truncation of the protein [26,27]. Since immunotherapeutic efficacies were still observed in these complement 5 deficient MMTV-PymT mice [15], the results suggest that complement 5 may not be involved in the pathway. Our studies collectively suggest there are multiple mechanisms operating in vivo. On one hand, the oncoproteins can be recognized either intracellularly or extracellularly after externalization by unconventional secretion, cell lysis, or being displayed as part of major histocompatibility complex (MHC) molecules. On the other hand, specific antibody–antigen interaction will ultimately trigger the host immune system which may engage

Table 1
Using 8 different mouse strains, the results illustrate that the therapeutic efficacy (+) depends on the presence (+) of B-cells.

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>T-cells</th>
<th>B-cells</th>
<th>Therapeutic efficacy</th>
<th>Number of mice used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nude</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>2364</td>
</tr>
<tr>
<td>Scid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>98</td>
</tr>
<tr>
<td>NOD-Scid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>40</td>
</tr>
<tr>
<td>C57BL6</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>688</td>
</tr>
<tr>
<td>muMT</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>50</td>
</tr>
<tr>
<td>Rag 2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>MMTV-PymT FVB/Nj</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>244</td>
</tr>
<tr>
<td>BALB/c</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>575</td>
</tr>
</tbody>
</table>
NK cells, B cells or T-cells. In summary, specific interactions between intracellular oncoproteins with antibodies will trigger a range of host immune responses that collectively manifest the therapeutic effect on cancer cells.

3. Clinical perspectives

3.1. Select 'tumor specific' therapeutic targets for immunotherapy

One of the most crucial challenges in the development of anti-cancer treatments is to find a specific 'tumor antigen' that is suitable for immunotherapy. It is essential to predict potential toxicity by carefully analyzing the expression levels of the candidate 'tumor antigen' in normal tissues. A desirable 'tumor antigen' should be highly expressed in cancer cells relative to normal cells, as ideal anti-cancer therapeutic agents should specifically target cancer cells while leaving normal tissues unharmed. Currently, only a few limited cell surface proteins have been selected for antibody therapies. Our unconventional concept prompts an evaluation of a wide spectrum of tumor-specific intracellular oncoproteins (including mutated oncoproteins, such as Ras and EGFR or viral proteins) as possible targets for mAb therapy or vaccination for human diseases. Oncogenic mutations commonly contribute to multiple human cancers and are often detected in intracellular proteins or the intracellular domains of cell surface proteins. Our recent research suggests an unconventional concept that intracellular oncoproteins can be targeted by therapeutic antibodies or peptide vaccination [15]. Generating antibodies against those mutations could specifically target cancer cells expressing respective mutated targets while sparing normal tissues. So far, hundreds of possible oncotargets have been found to have one or more mutations, which are listed at this website: http://share.gene.com/mutation_classification/cancer.variants.txt. Making antibodies one by one in vitro specifically against each point mutation is impractical, especially considering the high costs for existing antibody therapy.

Since modern technology can easily identify patients whose tumors are associated with a specific oncogenic mutation, peptides corresponding to mutated epitopes could be designed. An animal model is essential to pre-clinically evaluate the immunogenicity for each peptide. Vaccines can elicit long-lived immunity where the tumor specific antigens (or recombinant peptides) are used to trigger patients' immune system to make their own antibodies and activate cytotoxic T lymphocytes (CTLs) against mutated targets. Targeting oncoproteins with specific mutations is a precise approach against cancer cells since the mutated proteins only specifically exist in cancer cells but not in normal cells/tissues, hence minimizing side effects. Such epitope-based peptide vaccination is more rapid and economical than antibody development.

Our untraditional concept of immunotherapy presents a more specific strategy of targeting internal cellular proteins than using small-molecule inhibitors. The immunotherapies would target intracellular targets not only in cancer cells but also in cancer stem cells. Since cancer stem cells play important roles in disease recurrence and metastatic spread, targeting cancer stem cells will be particularly effective in treating cancer. As oncogenic mutations in stem or progenitor cells are believed to be part of the process in the development of cancer stem cells, antibodies against mutate oncoproteins such as K-Ras G12 V, G13D, and EGFR L858R will likely be able to target cancer cells and cancer stem cells [28,29] but keeping host normal tissues unharmed. The effect of intracellular tumor-specific antibodies on cancer stem cells may lower the rate of disease recurrence or metastatic spread.

If one looks past the long-standing dogma that antibodies only react against extracellular antigens, one would immediately appreciate a vast new array of intracellular oncoproteins as possible cancer therapeutic targets, thus realizing the full potential immunotherapies against both extracellular and intracellular oncotargets. Herein, we anticipate exploring buried intracellular treasures and vastly expanding the list of intracellular targets as candidates for immunotherapy.

3.2. The applications of antibody therapeutics

Metastasis confers a poor prognosis to cancer patients. Antibody therapies provide specific treatments against specific oncotargets. We proposed that antibodies are unlikely to be able to penetrate mature primary tumors which are generally protected with capsules (Fig. 2A), yet the antibody therapies are possible to markedly reduce metastatic tumor. This is because antibody
therapies may destroy invisible micro metastases (Fig. 2B) or circulating cancer cells (Fig. 2C). Oncologists can remove visible/mature primary tumors by surgery. We suggest that introducing antibodies into patient’s circulation (Fig. 2C) before or after surgery would be the key to reduce the risk of cancer metastasis and relapse. It is anticipated that the PRL-3 humanized antibody is likely to have broad applications in limiting the progression of different types of PRL-3 positive cancers, particularly in malignancies such as gastric cancers, lung cancers and AML which often relapses quickly. Clinical benefit will be more easily observed in more aggressive malignancies in initial clinical studies of PRL-3 positive patients.

Although our understanding of how PRL-3 antibodies inhibit PRL-3-positive tumors in vivo is not well understood, it should not deter clinical usage. Many drugs, including trastuzumab for breast and esophagogastric cancers, are used successfully in clinical practice without detailed understanding of their mechanisms of action.

### 3.3. Vaccine can be more prominent than antibody therapy

Existing antibody therapy for cancer treatment is very costly. We hope that our research will pave the way for cancer vaccination to become a mainstream cancer treatment that will be both effective and affordable. In patients with a strong family history of cancer, immunization of young susceptible family members with an antigen associated with the familial cancer could prime the immune system against tumor cells expressing that antigen and may prevent cancer before it develops.

### 3.4. Drug development prioritization

Unfortunately, cancer immunotherapy and vaccine development is limited by many factors, in particular funding constraints. Nearly any mutated or abnormally expressed protein in cancer cells can potentially serve as therapeutic targets, and so the number of possible targets dwarfs the key antigens that can be of current research interest. It is important to prioritize research targets and opportunities to maximize the benefit achievable with limited resources. The National Cancer Institute has conducted an immunotherapy agent workshop in 2007, ranking agents based on their resources. The National Cancer Institute has conducted an immune system against tumor cells expressing that antigen and may prevent cancer before it develops.

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### References


